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Dehydrogenation, oxidative denitration and ring contraction of *N*,*N*-dimethyl-5-nitrouracil by a *Bacillus* nitroreductase Nfr-A1

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ABSTRACT

Nfr-A1, a *Bacillus subtilis* nitroreductase, catalyzes the nitroreduction of a large panel of aromatic and heterocyclic nitro compounds, except those belonging to nitrouracil class of molecules. Besides nitroreduction, Nfr-A1 exhibits a strong NADH oxidase activity in the presence of oxygen, leading to high concentration of H_2O_2 (up to 200 μM). In the presence of (*N*,*N*)-dimethyl-5-nitrouracil **1** (dim-NU), Nfr-A1 achieves the reduction of dim-NU double bond to compounds **2** and **3** and in parallel the oxidation of dim-NU to the denitrated five membered derivatives **4** and **5**. The reduction is catalyzed by the reduced flavin Fl-$_{Red}$ and resembles those catalyzed by dihydropyrimidine dehydrogenases (DPD), during the catabolism of pyrimidines. The oxidative denitration is catalyzed in part by hydrogen peroxide generated through the NADH-oxidase activity, and certainly by the peroxyflavin intermediate Fl-$_{OOH}$ for the other part. The mechanisms of reaction were proposed according to experimental data and literature. These findings together with our previous results on the potential biological role of Nfr-A1, confirm the large spectrum of catalysis supported by this enzyme. The oxidative denitration is sporadically reported in literature and represents a safe and green alternative for the remediation of nitro-compounds.

1. Introduction

Nitroreductases are members of NAD(P)H/FMN oxidoreductases family. This appellation refers to their xenobiotic nitro substrates rather than their biological role witch is mostly unknown. The basis for their interest as pharmaceutical targets lies in the cell self-suicide, caused by the production of hydroxylamine intermediates that can be further metabolized to form cytotoxic DNA cross-linking agents. This property was exploited to design nitrofuran and nitroimidazole antibiotics [1]. Nitroreductases were also developed for enzyme/prodrug activation. The successful example is CB1954 that find applications in cancer prodrug therapy including antibody or gene-directed enzyme prodrug therapy [2] and Clostridia-directed enzyme prodrug therapy (CDEPT) [3]. Nitroreductases are involved in the activation of hypoxic anticancer nitro drugs in solid tumors [4]. The second application of nitroreductases is in the field of environment. These enzymes are involved in bioremediation of nitro-aromatic and nitro-heterocyclic pollutants, and were used for enzyme-based biosensor for nitro-sensitive ammunition [5,6].

Various known reductases are able to catalyze nitroreduction according to their sensitivity to oxygen. Type I or oxygen-insensitive nitroreductases catalyze the two-electron reduction of nitroaromatics. They include mammalian DT-diaphorase [7], xanthine dehydrogenase [8] and quinine reductase [9]. Type II or oxygen-sensitive nitroreductases includes various cytochrome reductases [10], ferredoxin and thioredoxin [11] and ubiquinone reductases [12]. These nitroreductases catalyze in the presence of oxygen the single-electron reduction of the nitro group to the nitro anion radical. This intermediates reacts with oxygen to generate superoxide, hydrogen peroxide and hydroxyl radicals. Some enzymes, initially classified as nitroreductases, catalyze the reduction of Cr(VI) to the less toxic Cr(III) compounds [13] and the reduction of various azo dyes in the presence of additional external FMN [14].

We have recently shown that *Bacillus subtilis* nitroreductase Nfr-A1 exhibits a strong NADH oxidase activity, catalyzes the degradation of the nicotinamide cofactor NAD^+ and scavenges high concentrations of H_2O_2 [15].

In order to further explore the catalytic potential of Nfr-A1, a large screening of diverse nitro substrates revealed that the nitro group of nitrouracil derivatives is not enzymatically reduced (results not shown). However, (*N*,*N*)-dimethyl-5-nitrouracil undergo a variety of unexpected reactions catalyzed directly or indirectly by Nfr-A1 and consisting of dihydrogenation, oxidative denitration and ring contraction.

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2. Experimental

2.1. Reagents

All chemicals were of the highest grade commercially available and purchased from Sigma–Aldrich, Fluka, or Merck. Parabanic acid is from TCI-Europe N.V., Belgium. Analytical grade solvents were from SDS (Peypin, France). Formic acid is HPLC-grade suprapur (Merck). (^{14}C)-methyl iodide was purchased from Amersham-Pharmacia-Biotech (Saclay, France). Superoxide dismutase (SOD, 4400 U/mg solid) and catalase (15,300 U/mg) were from Sigma–Aldrich.

2.2. N-methylation general procedure

This reaction was applied to uracil, 5-nitrouracil, 5-fluorouracil, parabanic acid and dialuric acid [24]. The desired quantity of the starting compound is solubilized in dimethylformamide DMF, and the solution stirred in ice under nitrogen atmosphere. 6 equiv. of NaH were added dropwise and allowed to react for 4 h. 2.5 equiv. of methyl iodide were then added and temperature rised to room temperature under stirring. The reaction is monitored by TLC or HPLC and stopped after total consumption of the starting material (3 h to overnight). DMF is evaporated under vacuum and the residue purified by flash chromatography on silica gel. Yields range from 65 to 90%.

N-N-dimethyl-5-nitrouracil ***1***: ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 8.72 (s, 1H), 3.59 (s, 3H), 3.38 (s, 3H). ^{13}C-NMR (62.5 MHz, $CDCl_3$): δ = 154.5, 150.0, 147.2, 125.2, 38.7, 29.0, MS (ES): m/z = 185.

N-N-dimethyl-parabanic acid: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.13 (s, 6H). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ = 160.4, 157.5, 28.43. MS (ES): m/z = 142.

2.3. Synthesis of 5-nitro-N,N-(^{14}C)-dimethyluracil

[^{14}C] methyl iodide (15 mCi, 55 mCi/mmole, from Amersham) was gauged into a vacuum ramp to afford 0.22 mmol of radioactive methyl iodide, i.e. 12.1 mCi. Non-labeled (0.56 mmol) methyl iodide in the gaseous form was mixed into the vacuum ramp with the radioactive methyl iodide to obtain an isotopic dilution to 15.4 mCi/mmol (0.78 mmol–12.1 mCi).

The [^{14}C] methyl iodide (12.1 mCi–15.4 mCi/mmol–0.78 mmol) was transferred under vacuum on a mixture of 53 mg (80% purity – 1.8 mmol) of sodium hydride, 45 mg (0.28 mmol) of nitrouracil in 5 mL of anhydrous dimethylformamide. The vacuum was broken under dry gaseous nitrogen and the mixture was allowed to react under stirring at room temperature during 5 h. The solution was evaporated and the residue purified.

Chromatography on silica gel offered 4.7 mCi of pure [^{14}C] dimethyl nitrouracil (radioactive yield: 39%). Chemical (98.7%) and radiochemical (96.1%) purities were determined by HPLC. Structure was assigned by NMR and mass spectrometry.

Specific activity was 27 mCi/mmol as measured by mass spectrometry and UV spectrophotometry. Stock solutions were prepared at 0.1 mCi/mL methanol (MeOH). Radiochemical purity was determined by HPLC. The original procedure is available in the lab book CEA/DSV/390 and the original spectra in the analysis report of CMM-2150.

2.4. Nitro-reduction of dim-NU

55 mg (0.3 mmol) of dim-NU were dissolved in 10 mL of MeOH then 15 mg of palladium on carbon catalyst (Pd/C) were added in a vessel equipped with a septum. Hydrogen was allowed to bubble into the mixture via a needle 2 h at atmospheric pressure and room temperature. The mixture is then filtrated and the solvent removed under reduced pressure. The product was purified by preparative thin layer silica gel chromatography to offer 26 mg (62% yield). ^{1}H NMR (300 MHz, CD_3OD): δ = 6.61 (s, 1H), 3.34 (s, 3H), 3.30 (s, 3H). ^{13}C NMR (62.5 MHz, CD_3OD): δ = 161.2, 150.4, 145.2, 121.5, 36.8, 28.4. m/z = 155 [MH^+].

2.5. Reduction of dim-NU double bond

500 mg (2.7 mmol) of dim-NU were dissolved in 50 mL of methylene chloride and 1 mL MeOH. 450 mg of $NaBH_4$ were added dropwise in ice and the reaction monitored by TLC. The reaction is stopped by the addition of water, and the compound extracted in dichloromethane. 370 mg of the ketone **2** were obtained from flash chromatography (75% yield). **2**: ^{1}H NMR (300 MHz, CD_3OD): δ = 5·24 (t, J = 4.7 Hz, 1H), 3.95 (q, J = 13.9 Hz, 1H), 3.94 (q, J = 13.9 Hz, 1H), 3.22 (s, 3H), 3.05 (s, 3H). ^{13}C NMR (62.5 MHz, CD_3OD): δ = 160.1, 152.1, 81.3, 46.9, 36.2, 28.7. MS (ES): m/z = 187.

Ketone **2** was stirred in distilled water overnight. 12 mg of the enol **3** were obtained by preparative HPLC. **3**: ^{1}H NMR (300 MHz, CD_3OD): δ = 4.15 (q, J = 14.4 Hz, 1H), 3.20 (s, 3H), 3.08 (s, 3H). ^{13}C NMR (62.5 MHz, CD_3OD): δ = 164.8, 156.0, 83.0, 48.4, 38.1, 30.8. MS (ES): m/z = 187.

2.6. Synthesis of 5-hydroxy-N,N-dimethyluracil

Methylation of 5-hydroxyuracil according to the procedure reported above lead to the trimethylated derivative 5-methoxy-*N*,*N*-dimethyluracil. The methoxy group was selectively demethylated as follows: 132 mg of 5-methoxy-*N*,*N*-dimethyluracil were dissolved in 3 mL of methylene chloride and mixed with 15.5 mL of 1 M solution of BBr_3 in methylene chloride. The mixture was allowed to react at 4 °C until total disappearance of the starting material in TLC, then the pH is adjusted to 9 with NH_4OH and the solvent evaporated. The mixture is recovered in 100 mL water and extracted with 6 × 50 mL of methylene chloride. 5-hydroxy-*N*,*N*-dimethyluracil was purified by flash chromatography in 75% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.85 (s, 1H), 3.39 (s, 3H), 3.36 (s, 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ = 161.4, 150.5, 131.8, 121.5, 37.2, 28.7. MS (ES): m/z = 185.

2.7. Reduction of N,N-dimethyl-parabanic acid to N,N-dimethyl-hydroxyhydantoin

140 mg (1 mmol) of *N*,*N*-dimethyl-parabanic acid were dissolved in 20 mL of methanol and the solution cooled in ice. 40 mg of $NaBH_4$ (1 equiv.) were added dropwise and the reaction closely monitored by HPLC to avoid any formation of the diol. The reaction is stopped by the addition of water, and the mixture evaporated. 52 mg of pure *N*,*N*-dimethyl-hydroxyhydantoin were obtained through flash chromatography.

N-N-dimethyl-hydroxyhydantoïn ***4***: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.25 (s, 1H), 3.02 (s, 3H), 2.99 (s, 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ = 173.3, 157.2, 79.6, 26.4, 24.5. MS (ES): m/z = 144.

2.8. Synthesis of N,N-dimethylhydantoin

N,*N*-dimethylhydantoin was synthesized according to the literature [16]. 176 mg (2 mmol) of dimethyl-urea and 200 mg of glyoxal (2 mmol) were mixed in pH 4 aqueous solution and held to react at 50 °C for 4 h. The solution is evaporated by vacuo, recovered in 20% $NaHCO_3$ and heated to 60 °C for 4 h. The mixture was evaporated by vacuo and extracted by ether. Ether was removed and the residue purified by flash chromatography to offer 115 mg of *N*,*N*-dimethyl-hydantoin.

N-N-dimethyl-hydantoin ***5****:* ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.85 (s, 2H), 2.97 (s, 3H), 2.98 (s, 3H). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ = 171.2, 157.9, 51.39, 26.5, 23.7. MS (ES): m/z = 128.

2.9. Conversion of dim-NU by Nfr-A1

Nfr-A1 was produced according to reference [16]. Activity was determined either by nitrite measurement, or HPLC–MS analysis of the produced metabolites. The reaction mixture consists of 66.7 μM of Nfr-A1, 1 mM of dimethyl-nitrouracil and 2 mM of NADPH in ammonium bicarbonate 50 mM pH 8.4. The reaction is placed at 37 °C. Incubation in partial anaerobia was conducted in 2 h nitrogen bubbled buffer containing NADH and dimethyl-nitrouracil. The mixture is placed at 37 °C under continuous vacuum for 30 min, then the nitroreductase is added by a syringe. For HPLC analysis, incubation samples were filtered on Vivaspin 500 tubes, with a 10 kDa molecular cutoff. For radioactive experiments, (^{14}C)-dim-NU is added to the incubation at the final radioactive concentration of 10^{-3} μCi mL^{-1}.

Preparative incubation was conducted on 50 mg of dim-NU. After completion, the reaction mixture was lyophilized and the residue recovered in 1 mL of methanol. Compounds were purified by preparative HPLC as reported bellow to give 10 mg of nicotinimide, 3 mg of **2**, 4 mg of **3**, 2.6 mg of **4** and 3.2 mg of **5**. Their structural data were identical to those of their synthetic counterpart presented above.

2.10. Nitrite measurement

Nitrite production was determined colorimetrically with the Griess reagent. 50 μL of the sample were mixed with 50 μL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine, 2.5% phosphoric acid) in 96-well microtiter plates. The absorbance at 550 nm was recorded on ELISA plates reader (Dynatech), and nitrite concentration deduced from a linear standard plot.

2.11. Hydrogen peroxide monitoring

Hydrogen peroxide production was determined colorimetrically by using the PeroXOquant Quantitative Peroxide Assay Kits (Pierce, Rockford, IL)

2.12. HPLC, HPLC–MS, and radioactivity counting

Analytical HPLC. High performance liquid chromatograph consists of an Alliance 2695 module and a 996 photodiode array detector (PDA) both controlled by a Millenium software (Waters corporation). The column used is a Hypercarb 5 microns, 100 × 4.6 mm id (ThermoQuest)., eluted at 1 mL/min with a linear gradient from water (0.1% formic acid) to acetonitrile (0.1% formic acid) in 50 min. Radioactivity was measured using an online Berthold LB506 C-1 HPLC detector managed by Winflow software.

Preparative HPLC. The chromatograph consists of a W600 pump, a 2487 dual detector set at 210 and 250 nm and a rheodyne manual injector equipped with a 10 mL loop. Operations are under the control of the Millenium software. The column is a preparative Hypercarb 7 microns, 150 × 10 mm id (ThermoQuest) eluted with the same gradient than analytical condition and operated at 7 mL/min.

HPLC–MS analysis. LC–ESI-MS analysis were performed on a simple-stage quadripole Waters-Micromass®ZQ 2000 mass spectrometer equipped with ESI (electrospray ionization) interface coupled to an Alliance Waters 2695 HPLC instrument with PDA and ELS detection. Mass spectrometry conditions were as follows: cone voltage, 30 V; source temperature, 150 °C; desolvation gas flow, 400 L/h; probe temperature, 450 °C; Corona current, 7 μA.

Radioactivity measurement. 20 μL of the incubation medium is mixed with 10 mL of aqueous scintillation cocktail (Aquasafe 300 plus from Zinsser Analytic). The label is counted in a LKB 1214 Rackbeta counter. Values correspond to the mean of three samples ± one standard deviation.

2.13. Nuclear magnetic resonance (NMR)

All type of NMR analysis were recorded in a Bruker 300 MHz or 500 MHz spectrometer (Bruker, Wissembourg, France), in $CDCl_3$, CD_3OD or DMSO as indicated.

3. Results and discussion

We recently exploited nitroreductase Nfr-A1 from *B. subtilis* in biosensing technology [6] and showed that this enzyme exhibits a powerful NADH-oxidase activity that account for diverse potential biological roles [15]. We also pointed out that the oxidative potential of this enzyme is more correlated to the peroxyflavin intermediate rather than the hydrogen peroxide formed. In order to detect side reactions revealing the catalytic potential of nitroreductases, we undertake a large screening of diverse nitro substrates. Among all series tested (Fig. 1), the nitro group of nitrouracil derivatives is not enzymatically reduced, although it easily forms the corresponding primary amine through catalytic hydrogenation. The appropriate substrate to assess the outcome of

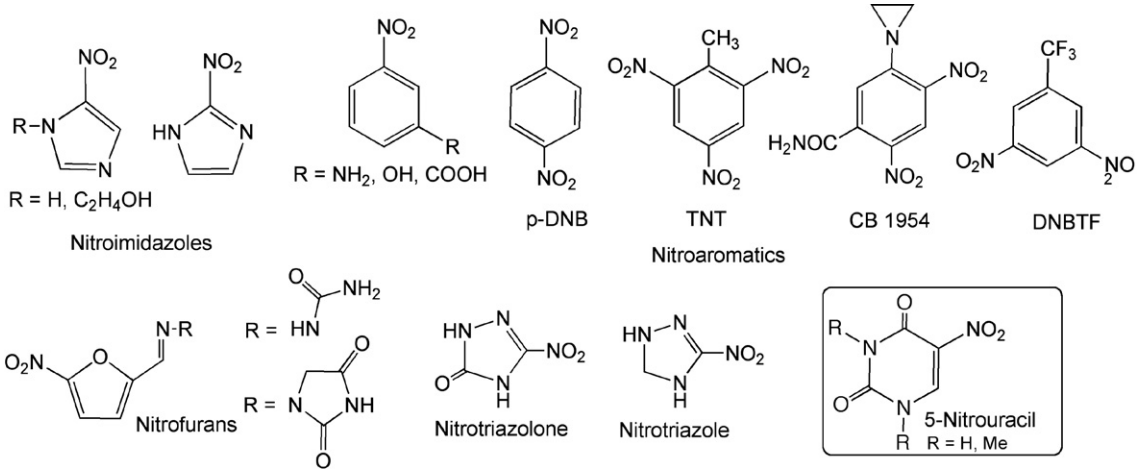

Fig. 1. Except the nitrouracil derivatives, these substrates undergo nitro-reduction by Nfr-A1 in the presence of added NADH.

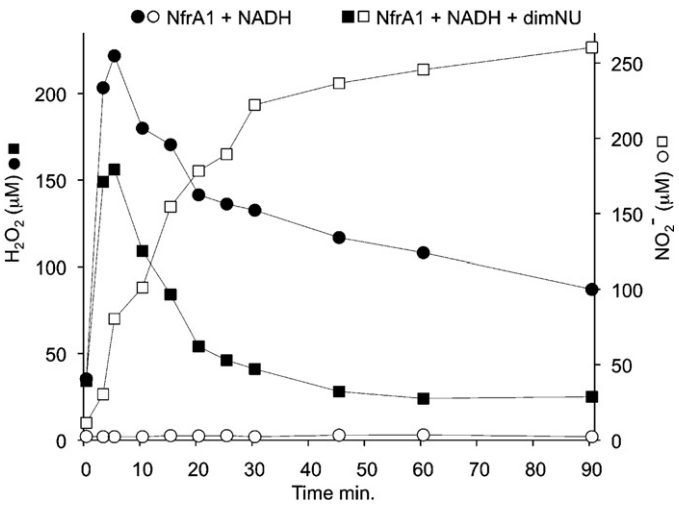

Fig. 2. Kinetics of production of nitrite and hydrogen peroxide by Nfr-A1 (□, nitrites and ■, hydrogen peroxide formed during the incubation of Nfr-A1 in the presence of NADH and DimNU; ○, nitrites and ●, hydrogen peroxide formed during the incubation of Nfr-A1 in the presence of NADH without the substrate DimNU).

nitrouracil derivatives is *N*,*N*-dimethyl-5-nitrouracil **1** (dim-NU). The *N*-alkylation prevents incorporation of the potential derivatives in the metabolism of uracil and enable radiolabelling through *N*-methylation. Thus, we synthesized the ^{14}C-labeled analog and apply combined UV-radioactivity HPLC to detect related metabolites (see Section 2 for details). We also monitored nitrites and hydrogen peroxide. Superoxide ion and hydroxyl radical were not formed in this reaction [15]. Fig. 2 shows that in the absence of the substrate **1** no nitrites were detected while hydrogen peroxide is formed as expected and reached 221 μM in 5 min. In the presence of dim-NU **1**, H_2O_2 did not exceed 156 μM in 5 min and quickly decreased to stabilize around 25 μM in 45 min. Nitrites release is inversely proportional to H_2O_2 production and reached a maximum of 260 μM in 90 min. These results indicate that Nfr-A1 catalyze efficiently the denitration of dim-NU.

Enzymatic denitration is sporadically reported in literature, mainly for energetic compounds and consisted of C-, N- or O-denitration. This reaction finds application in both therapeutic and environmental fields. In the field of bioremediation, it includes the enzymatic denitration of organic nitrate esters (trinitroglycerin (TNG), pentaerythritol tetranitrate (PETN), nitrocellulose, nipradilol), of nitramines (royal demolition explosive RDX, high melting point explosive HMX, hexanitro-hexaazaisowurtzitane CL20, tetryl), nitro-aromatic compounds (trinitrotoluene TNT, picric acid, nitrotyrosine) and nitroheterocyclic compounds (nitracrine, quinifuryl, nitrotriazolone (NTO)). Thus, glutathione-*S*-transferase [17], DT-diaphorase [7], cyt-P450 [10] and various oxido-reductases were reported to catalyze such a reaction [18–21]. The denitration of nitrate esters and nitroheterocycles is closely correlated to the production of nitric oxide that accounts for the vasorelaxant therapeutic effect of these compounds [22]. To our knowledge, the enzymatic denitration of nitrouracils has never been reported before.

HPLC chromatogram presented in Fig. 3 was obtained after 1 h of incubation and shows five new peaks. Peaks **2**, **3**, **4** and **5** are ^{14}C-labeled and subsequently belongs to dim-NU while the fifth peak correspond to free nicotinamide obtained through the previously reported NAD^+ oxidation [15]. After 3 h of incubation, the conversion of dim-NU is complete, with only slight evolution in peaks proportion. Filtration on 1 kDa molecular cutoff membranes, that totally retains the protein, shows that the entire radioactivity is recovered in the filtrate. This indicates the absence of any substrate/enzyme complex.

In order to achieve structural elucidations, a preparative incubation of 50 mg of dim-NU was engaged, and compounds were purified by preparative HPLC. ^{1}H-NMR, ^{13}C-NMR and mass spectrometry investigations indicate that compound **2** at retention time 19.7 min results from the enzymatic reduction of the pyrimidine double bond. This ketone **2** tautomerize to the corresponding enol **3** offering the peak at Rt 18.8 min. In order to confirm these structures, dim-NU **1** was reduced by $NaBH_4$ to ketone **2**. Recording of

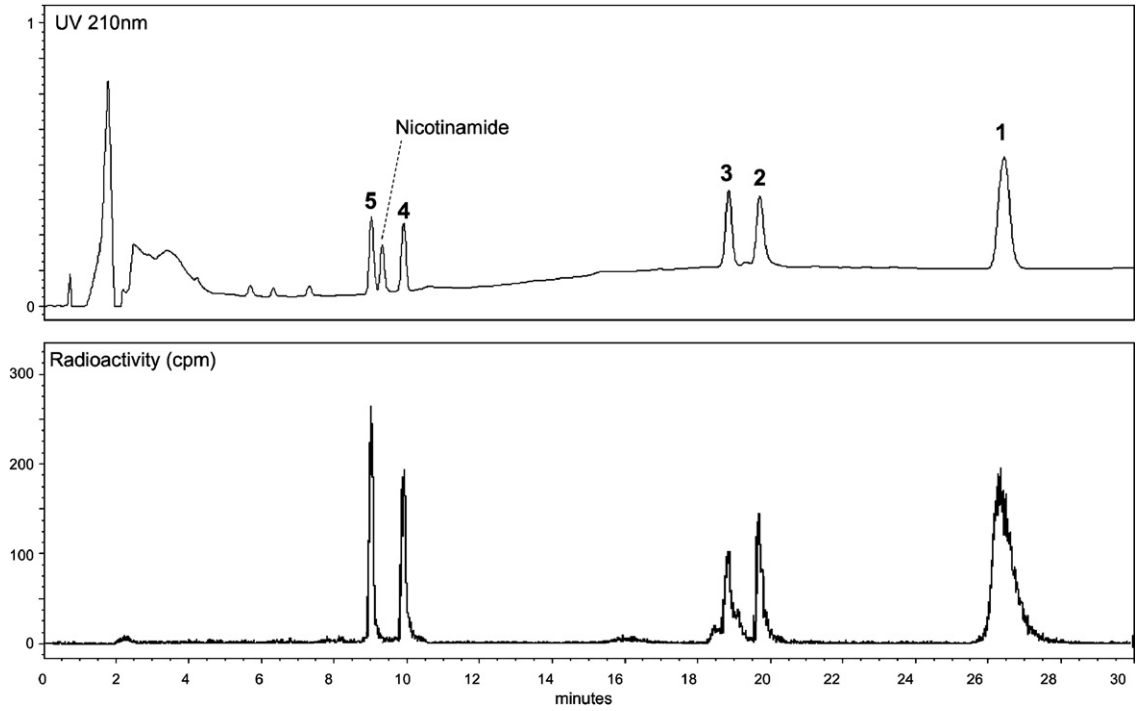

Fig. 3. HPLC analysis after 1 h of incubation of ^{14}C-labeled dim-NU with Nfr-A1. Chromatogram shows concomitant UV and online radiolabel measurement.

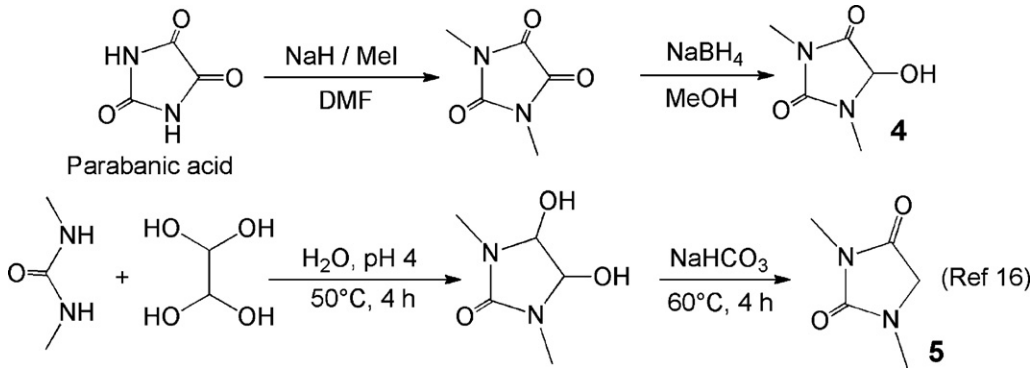

Fig. 4. Synthetic routes to the isolated metabolites **4** and **5**.

1H-NMR spectra in D_2O show that the proportion of the enol **3** peak increased with time to reach the 50/50 ratio observed in HPLC.

The HPLC peaks at retention time Rt 9 and 10 min have a molecular mass of 128 and 144 respectively. These pair masses attest for a loss of a nitrogen atom suggesting that these compounds arise from the denitration of dim-NU. Their structures were deduced from NMR and mass spectra. The peak having a molecular mass of 144 correspond to *N*,*N*-dimethyl-hydroxyhydantoin **4**, which is confirmed by comparison with an authentic sample obtained via controlled $NaBH_4$ reduction of *N*,*N*-dimethyl parabanic acid (Fig. 4). The compound with molecular mass 128 corresponds to *N*,*N*-dimethyl-hydantoin **5**. Authentic sample of this compound was prepared according to the literature (Fig. 4) [16].

3.1. Dihydropyrimidine dehydrogenase (DPD) like-reaction catalyzed by Nfr-A1

The first reaction catalyzed by Nfr-A1 is the reduction of the pyrimidine double bond of **1**. This reaction resembles those catalyzed by dihydropyrimidine dehydrogenases (DPD), during the catabolism of pyrimidines [23]. This extensively investigated enzyme is responsible for the resistance to anticancer 5-fluorouracil therapy, especially in DPD overproducing patients [24]. In these patients, the reduction of 5-FU double bond by DPD leads to a totally inactive derivative. Recent therapies have recommended the combination of 5-FU with the DPD inhibitor capecitabine [25]. Compared to Nfr-A1, DPD is a complex homodimeric protein containing two FAD, two FMN and eight [4Fe–4S] clusters [26], nevertheless the catalytic agent remains the reduced FMN.

In our conditions, Nfr-A1 failed to reduce 5-fluorouracil and *N*,*N*-dimethyl-5-fluorouracil (results not shown). This is probably due to the opposite electronic effects between the electron-attractive nitro group and the electron-withdrawing fluor atom that influence the reactivity of the double bond. As a confirmation, borohydride that efficiently reduces dim-NU double bond, failed in reducing dim-5-FU or 5-FU. Nevertheless, 5-fluorouracil reduction is still under debate in the literature [27].

Reduction of dim-NU by Nfr-A1 benefits from the conjugation of two favorable events, an electron-attractive nitro group at position 5, and alkylation of nitrogen at position 1. Thus, the electronic delocalization in the *N*-protonated uracil supported the prevalence of the enol form (Lactim) rather than the diketo form (Lactam). The impact of uracil tautomerism on it's reactivity is widely reported in literature [28,29]. This hypothesis is consistent with experimental data in witch Nfr-A1 failed to reduce *N*,*N*-dimethyluracil or nitrouracil double bond (data not shown).

3.2. dim-NU denitration and ring contraction catalyzed by Nfr-A1

The unexpected reactions are those involved in ring contraction of dim-NU that leads to compounds **4** and **5** and account for nitrite production. Incubation of authentic samples of these compounds with Nfr-A1 shows that they do not derive from each other suggesting parallel pathways.

In order to discriminate between a reductive or oxidative catalysis, the enzymatic reaction was conducted in partial anaerobia. In these conditions peaks **2** and **3** were not significantly affected while the proportion of peaks **4** and **5** as well as nitrite formation decreased by 70% (Fig. 5).

The first step in mechanism elucidation is the identification of the denitration substrate among dim-NU **1**, the ketone **2** or the enol **3**. Separate incubation of these compounds with Nfr-A1 shows that **2** and **3** neither undergoes denitration nor the subsequent production of **4** and **5**. Subsequently those latter derive directly from dim-NU **1**. As no equivalent enzymatic reaction is reported in the literature, we collected key information from similar chemical reactions. Few examples reported the denitration or ring contraction of uracil derivatives substituted at position 5. Most of them involve drastic oxidation reagents such as hydroxyl radical [30], ozone [31,32] or Br_2/O_2 [33] (Fig. 6). The authors suggested a putative instable six-membered intermediate, hydroxylated at position 6, that undergoes spontaneous ring contraction. Furthermore, H_2O_2 and other peroxides oxidize uracil in alkali through epoxide and diol intermediates [34].

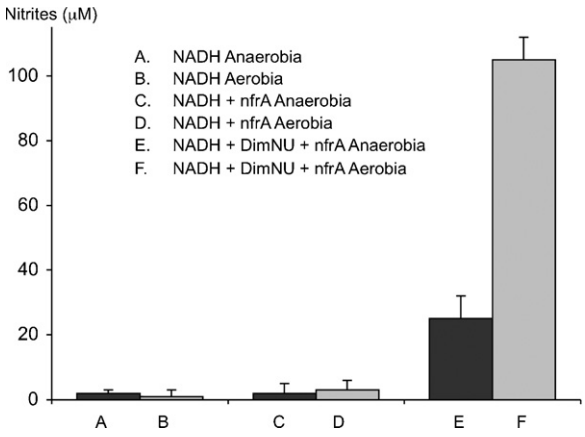

Fig. 5. Inhibition of nitrite production in partial anaerobia (column E compared to column F). (A) NADH control in partial anaerobia; (B) NADH control; (C) Nfr-A1 + NADH control in partial anaerobia; (D) Nfr-A1 + NADH control; (E) incubation of Nfr-A1 + NADH with the substrate DimNU in partial anaerobia; (F) incubation of Nfr-A1 + NADH with the substrate DimNU.

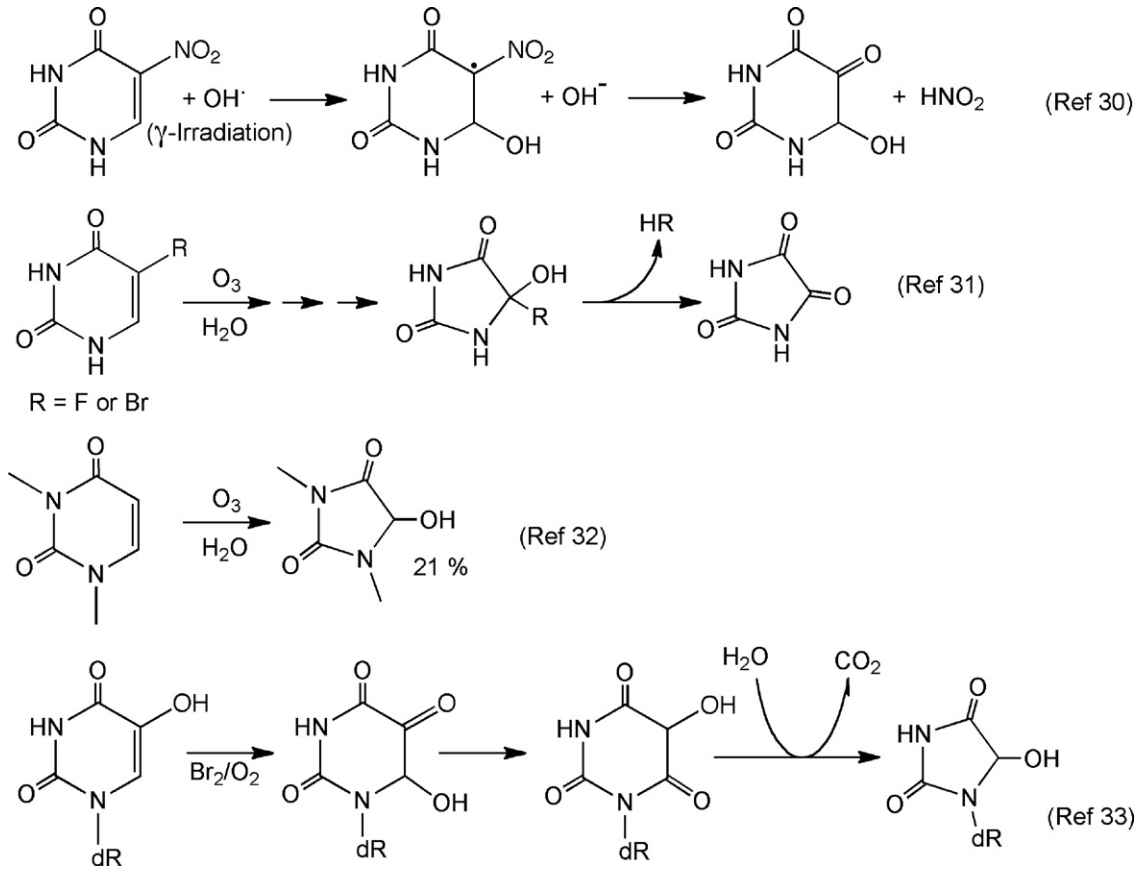

Fig. 6. Examples of chemical oxidative denitration and/or ring contraction of uracil derivatives involving highly reactive oxygen species.

We recently reported that Nfr-A1 produce hydrogen peroxide through an efficient NADH-oxidase activity [15]. In order to check for H_2O_2 involvement in dim-NU denitration, we investigated the impact of catalase and superoxide dismutase (SOD) on HPLC profile and nitrite production, as well as the reactivity between dim-NU and H_2O_2. Fig. 7 shows that SOD has no impact on HPLC profile, while catalase, that trap hydrogen peroxide, inhibit the formation of compound **4** but not **5**. In this case, dim-NU was not completely converted. After 3 h of incubation nitrites did not exceed 125 μM instead of 265 μM obtained in the absence of catalase.

Fig. 8A shows that H_2O_2 catalyze, in a dose dependent manner, the denitration of dim-NU at the pH of 8.4 used for the enzymatic reaction. The subsequent formation of compound **4** is confirmed by HPLC analysis (Fig. 8B). The reaction mechanism is certainly similar to equivalent case reported in literature mechanism [34]. In contrast with compound **4** issued from the oxidation of dim-NU by hydrogen peroxide, the formation of **5** involves different catalyst. As previously reported, Nfr-A1 did not produce neither

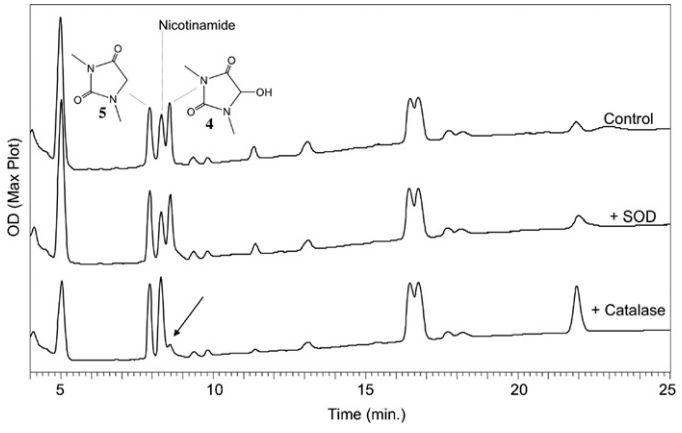

Fig. 7. Influence of catalase and superoxide dismutase SOD on HPLC profile. Addition of catalase inhibits the formation of compound **4** but not **5**.

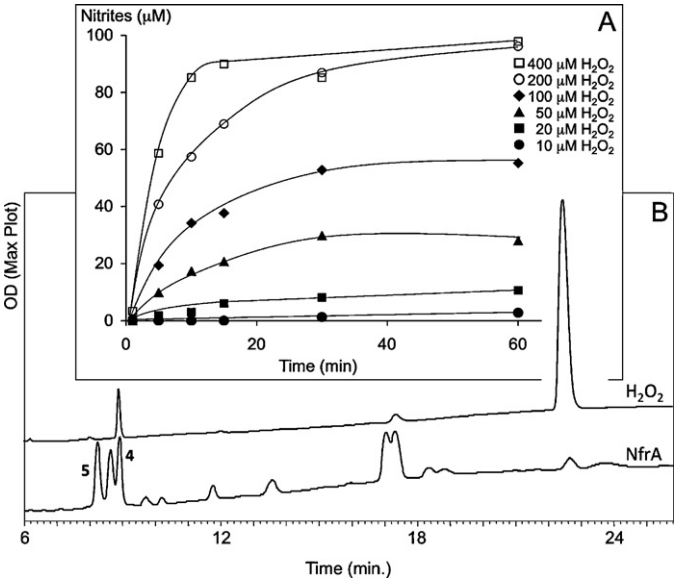

Fig. 8. Dose-dependent denitration of dimNU by hydrogen peroxide (A); symbols correspond to increasing concentrations of hydrogen peroxide in (●, 10 μM; ■, 20 μM; ▲, 50 μM; ♦, 100 μM; ○, 200 μM; □, 400 μM). Denitration leads to compound **4** but not **5** as confirmed by HPLC analysis (B).

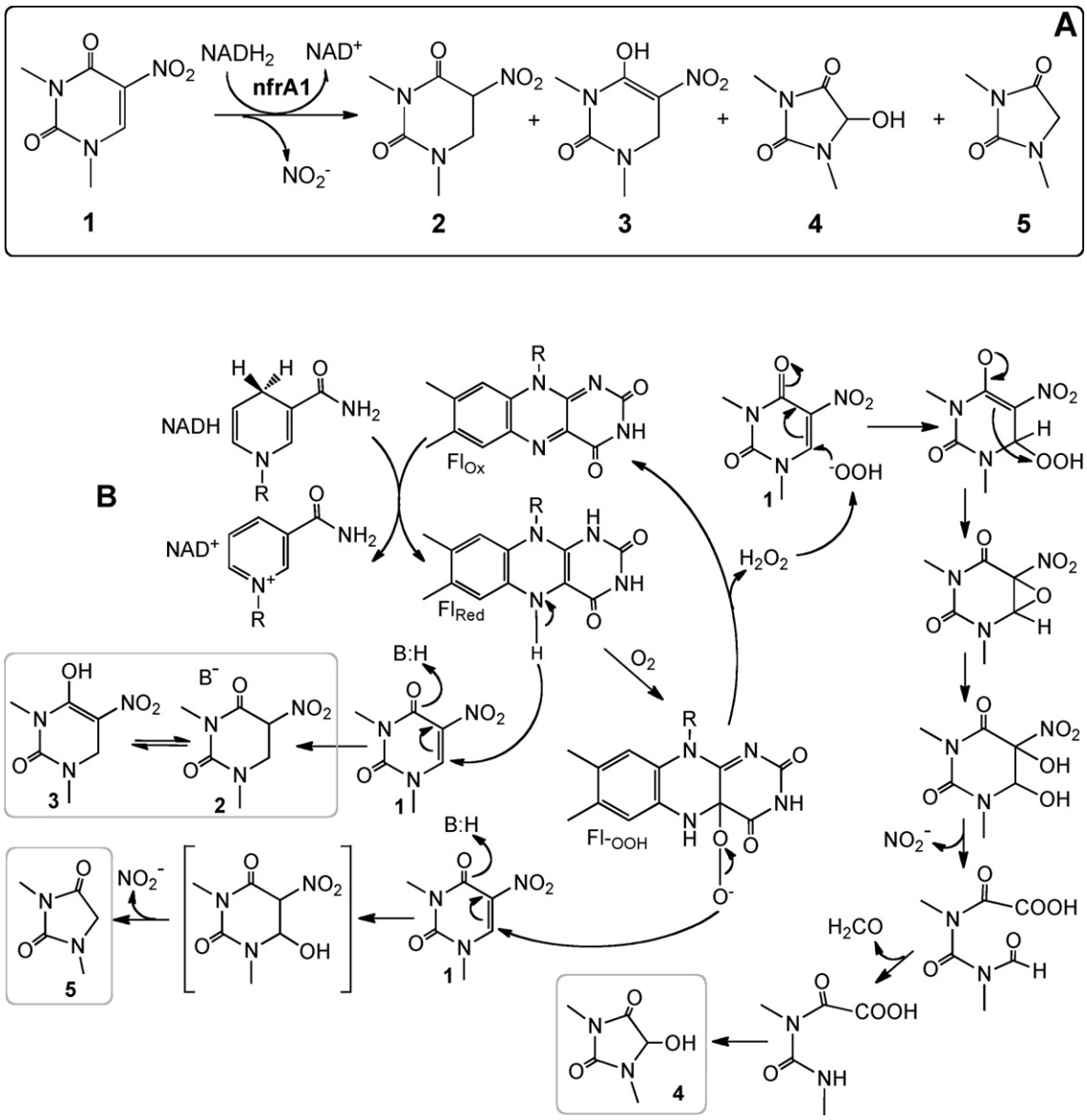

Fig. 9. Summary of the compounds formed (A) and proposed mechanisms of formation of the compounds identified in this study (B).

superoxide ion nor hydroxyl radical [15], the remaining oxidizing agent that could assume such a reaction is the peroxyflavin intermediate previously suspected for NAD^+ degradation [15], and reported recently for aromatic ring-cleavage reactions [35]. The summary of the reactions and the proposed mechanisms of formation of various derivatives are summarized in Fig. 9.

In conclusion we have recently shown that *B. subtilis* nitroreductase Nfr-A1 exhibits a strong NADH oxidase activity leading to high concentration of H_2O_2. The produced hydrogen peroxide accounts partially for the oxidative potential of Nfr-A1, besides the highly reactive peroxyflavin intermediate $Fl\text{-}_{OOH}$ formed by molecular oxygen interaction with the reduced flavin $Fl\text{-}_{Red}$ [15]. In this paper we confirm that Nfr-A1 can achieve the reduction of dim-NU double bond by the reduced flavin to **2** and **3** and in parallel the oxidation of dim-NU to the denitrated five membered derivatives **4** and **5**. The oxidative denitration is catalyzed in part by hydrogen peroxide generated though the NADH-oxidase activity, and certainly by the peroxyflavin intermediate $Fl\text{-}_{OOH}$ for the other part. These findings together with our previous results on the potential biological role of Nfr-A1, confirm the large spectrum of catalysis supported by this enzyme. We try now to enlarge the spectrum of reduction and oxidation to highly recalcitrant sulfur compounds such as thiophene and benzothiophene.

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