

# The enzymatic reduction of pyridine N-oxide derivatives: the role of their electron accepting potency

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The reduction reactions of a series of pyridine- and nitropyridine-N-oxide compounds by single-electron transferring flavoenzyme *Anabaena* PCC 7119 ferredoxin-NADP<sup>+</sup> oxidoreductase (FNR, EC 1.18.1.2) were examined. The steady-state bimolecular rate constants ( $k_{cat}/K_m$ ) of the reduction of pyridine N-oxides were determined to range between  $1.3 \times 10^1$  and  $2.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ . The quantitative structure activity relationships (QSARs) were defined between the logarithm of the  $k_{cat}/K_m$  parameter of the compounds' reduction and the energies of their lowest unoccupied molecular orbitals ( $E_{LUMO}$ ) obtained by quantum-mechanical methods. QSARs studies showed that the reactivity of pyridine N-oxide derivatives was generally higher than that of nitroaromatic model compounds. The pyridine N-oxide as well as nitropyridine N-oxide derivatives proved to be more efficient substrates for the single-electron flavin-dependent enzyme than the model nitroaromatics and thus can be attributed to a new series of closely related compounds. This preliminary study may serve as a useful tool for predicting the enzymatic reactivity of structurally related compounds and may be used for analysis of their cytotoxicity with respect to the possible involvement of their redox cycling.

**Key words:** pyridine N-oxides, lowest unoccupied molecular orbital, electron transfer, ferredoxin reductase, quantitative structure activity relationship

## INTRODUCTION

The application of N-oxide derivatives received considerable attention due to their usefulness as synthetic intermediates and biological importance. Heterocyclic N-oxides are also useful as protecting groups, auxiliary agents, oxidants, ligands in metal complexes and catalysts [1]. Pyridine N-oxides are intensively used as precursors for the synthesis of corresponding pyridines, tetrazolo[1,5-a]pyridines and stereodefined diene oximes compounds [2–4]. Pyridine N-oxides take part in reaction with Grignard reagents [2, 5] and are used as organocatalysts for the asymmetric allylation of aldehydes [6]. They can be functionalized by palladium or nickel catalyst [5, 7]. The oxidation of pyridines to their N-oxides is employed in drug discovery programs [8]. Pyridine N-oxide derivatives represent a new class of anti-HIV compounds, for

which some of the members exclusively act through inhibition of HIV-1 reverse transcriptase and thus behave as the non-nucleoside reverse transcriptase inhibitors [10]. The oxide part of the pyridine moiety proved to be indispensable for anti-coronavirus activity. The potency and virus specificity of the pyridine N-oxides were shown to vary depending on the nature and location of alkyl, halogen, nitro and other substituents on the molecule [10]. The carcinogenicity and mutagenicity of 4-nitropyridine N-oxide and its derivatives were tested on mice, *Salmonella typhimurium* and *Escherichia coli* strains. However, no clear quantitative relationship between mutagenicity and carcinogenicity was found among the compounds examined [11]. The Ames test of pyridine N-oxides on *Salmonella typhimurium* strains showed a mutagenic action without metabolic activation [12]. Some N-oxide derivatives may act as bioreductive drugs [13]. The toxicity of these compounds was found to be exerted *via* intracellular metabolism to highly toxic radicals. At a low oxygen level, N-oxide

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compound radicals react with cellular molecules, causing DNA damage and cell death. In aerobic cells, the toxicity of these compounds results from the back-oxidation of their radicals by molecular oxygen [13].

The objective of this work was to carry out a preliminary study of pyridine N-oxide derivatives as redox active substrates in reactions with *Anabaena* PCC 7119 NADP<sup>+</sup>-ferredoxin reductase (FNR, EC 1.18.1.2) used as a model electron transferring flavoenzyme. We have attempted to define the quantitative structure activity relationship (QSAR) between the enzymatic reactivity of compounds and their electron accepting potency expressed as the energy of the lowest unoccupied molecular orbitals. This may serve as a useful tool for predicting the reactivity of related compounds and may be used in future for the analysis of their cytotoxicity with respect to the possible involvement of their redox cycling.

## EXPERIMENTAL

**Chemicals and enzyme.** The structural formulas of pyridine N-oxide derivatives are presented in Fig. 1. 2,3-Dimethylpyridine N-oxide (1) was prepared from 2,3-dimethylpyridine using the oxidation procedure with hydrogen peroxide in acetic acid as described in [14] (m. p. 95–96 °C, <sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ(ppm): 2.29 (s, 3H), 2.45 (s, 3H), 6.94–7.04 (m, 2H), 8.09–8.13 (m, 1H)). Pyridine N-oxide (2) (m. p. 66–68 °C, <sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ(ppm): 7.30–7.37 (m, 3H), 8.23–8.31 (m, 2H)), 4-chloropyridine N-oxide (3) (m. p. 169–170 °C, <sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ(ppm): 7.23–7.33 (m, 2H), 8.11–8.20 (m, 2H)) and 4-nitropyridine N-oxide (8) (m. p. 160–162 °C, <sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ(ppm): 8.06–8.12 (m, 2H), 8.20–8.26 (m, 2H)) were synthesized by the methods reported in [15]. 4-Chloro-2,3-dimethylpyridine N-oxide (4) was obtained starting with 2,3-dimethyl-4-nitropyridine by reaction with hydrochloric acid

as described in [16] (m. p. 104–105 °C). <sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ(ppm): 2.35 (s, 3H), 2.51 (s, 3H), 7.11 (d, 1H J = 6.9 Hz), 8.06 (d, 1H J = 6.9 Hz)). 2,3-Dimethyl-4-nitropyridine N-oxide (5) was synthesized according to the described method [17] (m. p. 91–92 °C). <sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ(ppm): 2.57 (d, 6H J = 6 Hz), 7.70 (d, 1H J = 7.2 Hz), 8.20 (d, 1H J = 7.2 Hz)). 2-Chloro-3-methoxy-6-nitropyridine N-oxide (6) was prepared from 2-chloro-3-methoxypyridine N-oxide by the method described in [18] (m. p. 103–105 °C). <sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ(ppm): 4.12 (s, 3H), 7.83 (d, 1H J = 7.5 Hz), 8.23 (d, 1H J = 7.5 Hz)). 2-Bromo-3-methoxy-6-nitropyridine N-oxide (7) was obtained by a published method [18] (m. p. 140–142 °C). <sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ(ppm): 4.10 (s, 3H), 6.92 (d, 1H J = 9 Hz), 7.35 (d, 1H J = 9 Hz)). NADPH and cytochrome C were purchased from Reanal and Sigma, respectively. *Anabaena* PCC 7119 ferredoxin : NADP<sup>+</sup> reductase was prepared as described previously [19] and was a generous gift of Dr. Martinez-Julvez and Professor C. Gomez-Moreno (Zaragoza University, Spain). The enzyme concentration was determined according to the absorbance of FAD, ε<sub>459</sub> = 9.4 mM<sup>-1</sup> cm<sup>-1</sup> [20].

**Enzymatic assay.** The kinetic measurement of FNR-catalyzed reduction of pyridine N-oxides was carried out spectrophotometrically using a Hitachi-557 spectrophotometer in 0.1 M K-phosphate buffer containing 1 mM EDTA at 25 °C. The rates were monitored by following the oxidation of NADPH (Δε<sub>340</sub> = 9.4 mM<sup>-1</sup> cm<sup>-1</sup>). The rates obtained were corrected for intrinsic NADPH : oxidase activity of enzyme. To 2 ml of the reaction mixture, 10–20 μl solution of the pyridine N-oxide substrates dissolved in DMSO was added to start the reaction. The FNR concentration varied between 25 and 50 nM. As it was tested previously, 1–2% of DMSO in the enzyme reaction mixture do not influence the kinetic experiments [21]. In separate experiments, the reduction of cytochrome c (50 μM) in the presence of NADPH and pyridine N-oxide was monitored using Δε<sub>550</sub> = 19.2 mM<sup>-1</sup> cm<sup>-1</sup>.

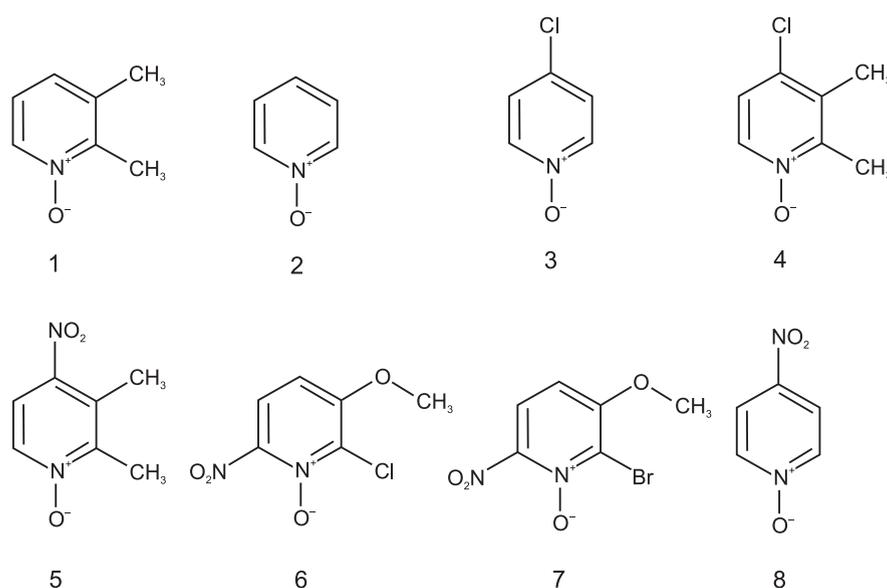


Fig. 1. Structural formulae of pyridine N-oxide compounds used in this study

Assuming a ping-pong reaction scheme for FNR [21], the kinetic parameters were determined at fixed concentration of NADPH (100  $\mu\text{M}$ ) and varied concentrations of pyridine N-oxide substrates. Typically, 6–8 concentrations of compounds were used. Due to a limited solubility or a high optical density of the compounds at their higher concentrations, a function of the following form was fitted to the experimental data [22]:

$$v / [E_0] = k_{cat} / K_{m(\text{PyNO})} \{ [\text{PyNO}] / (1 + [\text{PyNO}] / K_{m(\text{PyNO})}) \}, \quad (1)$$

where  $v$ ,  $[E_0]$  and  $K_{m(\text{PyNO})}$  respectively are the initial rate of NADPH oxidation, the total FNR concentration and the Michaelis–Menten constant for the reduction of pyridine N-oxide substrate  $[\text{PyNO}]$ . This function represents a reparameterized Michaelis–Menten equation which permits an accurate calculation of the  $k_{cat} / K_{m(\text{PyNO})}$  ratio even when the  $k_{cat}$  and  $K_{m(\text{PyNO})}$  parameter values cannot be determined separately [22]. In situations when  $[\text{PyNO}] / K_{m(\text{PyNO})} \ll 1$ , Eq. 1 can be transformed into a simplified form:

$$v / [E_0] = k_{cat} / K_{m(\text{PyNO})} [\text{PyNO}]. \quad (2)$$

**Quantum-mechanical calculations and statistical analysis.** Quantum-mechanical calculations of pyridine N-oxide compounds were performed using PC Spartan'04 (Wavefunction, Inc) software. For the initial refinement of the molecular geometry of compounds, the force field (MMFF) method of molecular mechanics was used. For the further geometry optimization and evaluation of the quantum chemical parameters of the compounds, the semi-empirical AM1 and PM3 methods were used. In this study, the outcomes of quantum-mechanical calculations on pyridine N-oxide molecules in vacuum are related to the electronic characteristics of the substrates in the active site of the enzyme. Due to solvation effects and different dielectric constants, the intrinsic properties of the compounds might be influenced upon binding to the active site. Nevertheless, it is assumed that this phenomenon will not have a substantial influence on the relative difference for a series of closely related compounds [23–25]. The computer calculation can thus be used as an approach to studying the relative differences within a series of related compounds or within one molecule.

The evaluation of experimental kinetic data and regression analysis of QSAR were performed using SigmaPlot 2000 (version 6.01) and Statistica (version 4.3; Statsoft, 1993) softwares, respectively.

## RESULTS AND DISCUSSION

The study of non-enzymatic single-electron reduction of several pyridine N-oxide derivatives by photo-excited NADH analogue 1-benzyl-1,4-dihydrocinchonamide (BNAH) as a model compound has been recently reported by Nikanishi et al. [26]. In our study, various structure pyridine N-oxide

compounds were used as substrates of the single-electron transferring flavoenzyme FNR, and the efficiency of their reduction was measured. It may be noted that these compounds were not used at sufficiently high concentrations for an accurate determination of  $k_{cat}$  and  $K_{m(\text{PyNO})}$  parameter values due to their poor solubility and / or high optical density at high concentrations. Nevertheless, the  $k_{cat} / K_{m(\text{PyNO})}$  values were determined accurately from Equations 1 or 2. It is commonly accepted that the  $k_{cat} / K_m$  ratio is a measure of catalytic efficiency and represents the substrate binding and potential for catalysis at a low substrate concentration, which reflects the *in vivo* condition [22, 27]. As determined in our previous studies [21, 28], the steady-state reduction of quinones and nitroaromatic compounds by FNR followed a classical ping-pong reaction scheme. The transient kinetics experiments of photo-reduced FNR reoxidation by 5,8-dihydroxy-1,4-naphthoquinone and 2,4,6-trinitrotoluene (TNT) showed that the bimolecular rate constants for these reactions were very close or equal to the  $k_{cat} / K_m$  values obtained by steady-state experiments [21, 29, 30]. The  $k_{cat} / K_m$  behaves as a bimolecular rate constant between the free enzyme and the substrate, and it is a useful index for comparing the relative activities. Therefore, the further analysis of enzyme reactivity was performed using this parameter. As one can see from Table, the  $k_{cat} / K_{m(\text{PyNO})}$  parameter values vary by several orders of magnitude.

In a separate experiment, we have tested whether pyridine N-oxide derivative can be a redox-cycling compound in a FNR-catalyzed reaction. We have found that the reduction of pyridine N-oxide compound by FNR was accompanied by its redox cycling, i. e. the oxidation of NADPH excess over compound concentration. The FNR-catalyzed reduction was accompanied by the reduction of cytochrome *c* (50  $\mu\text{M}$ ) added into the reaction mixture. The single-electron flux was estimated by 4-chloro-2,3-dimethylpyridine N-oxide mediated reduction of cytochrome *c*. The percentage of single-electron flux, expressed as the ratio between cytochrome *c* reduction rate and the double rate of enzymatic NADPH oxidation, equalled to  $94 \pm 5\%$  ( $n = 3$ ). This process was inhibited with 30  $\mu\text{g/ml}$  superoxide dismutase by 40–45%. This preliminary study demonstrates the possibility of involvement of the pyridine N-oxide anion radical and superoxide generation as a result of anion radical reoxidation by oxygen.

Subsequently, we analysed the possible relationships of the reactivity of pyridine N-oxide derivatives and their electron-accepting potency. Since the single-electron potentials ( $E^1$ ), pyridine N-oxides are not available, the energies of their lowest unoccupied molecular orbitals ( $E_{LUMO}$ ) were calculated by quantum-mechanical methods. Although  $E_{LUMO}$  are representatives of the relative ease of single- or two-electron reduction of compounds, our results have shown that FNR supports the redox cycling of pyridine N-oxides, suggesting that the enzyme catalyzes single-electron reduction of these substrates. Thus,  $E_{LUMO}$  of pyridine-N-oxides can be used as a measure of their single-electron accepting potency. As one can see in Table, the reactivity of pyridine N-oxide derivatives

Table. The bimolecular rate constants ( $k_{cat}/K_m$ ) of FNR-catalyzed reduction of pyridine N-oxide derivatives and nitroaromatic model compounds, and their  $E_{LUMO}$  values as calculated by semi-empirical AM1 and PM3 methods

No.	Compound	$k_{cat}/K_m$ ( $M^{-1} \cdot s^{-1}$ )	$E_{LUMO}$ (eV)	
			RHF / AM1	RHF / PM3
<i>Pyridine-N-oxides</i>				
1	2,3-Dimethylpyridine N-oxide	$1.3 \pm 0.1 \times 10^1$	-0.28	-0.53
2	Pyridine-N-oxide	$6.8 \pm 1.4 \times 10^1$	-0.33	-0.59
3	4-Chloro-pyridine-N-oxide	$2.4 \pm 0.4 \times 10^2$	-0.62	-0.79
4	4-Chloro-2,3-dimethylpyridine-N-oxide	$7.1 \pm 0.9 \times 10^2$	-0.51	-0.72
5	4-Nitro-2,3-dimethylpyridine-N-oxide	$3.7 \pm 0.9 \times 10^3$	-1.55	-1.38
6	2-Chloro-3-methoxy-6-nitropyridine N-oxide	$8.8 \pm 0.4 \times 10^3$	-1.28	-1.44
7	2-Bromo-3-methoxy-6-nitropyridine N-oxide	$1.2 \pm 0.2 \times 10^4$	-1.29	-1.41
8	4-Nitropyridine N-oxide	$2.0 \pm 0.3 \times 10^4$	-1.70	-1.61
<i>Nitroaromatic compounds</i>				
9	Nitrobenzene	$5.9 \pm 0.5 \times 10^1$	-1.07	-1.13
10	4-Nitrobenzyl alcohol	$1.4 \pm 0.1 \times 10^{2a}$	-1.20	-1.26
11	4-Nitrobenzaldehyde	$8.3 \pm 0.7 \times 10^{3b}$	-1.67	-1.69
12	1,2-Dinitrobenzene	$2.2 \pm 0.2 \times 10^{3b}$	-1.84	-1.85
13	1,3-Dinitrobenzene	$2.5 \pm 0.2 \times 10^{3b}$	-1.91	-1.96
14	1,4-Dinitrobenzene	$3.0 \pm 0.2 \times 10^{4b}$	-2.21	-2.25
15	2,4,6-Trinitrotoluene	$1.1 \pm 0.1 \times 10^{4c}$	-2.43	-2.45

<sup>a, b, c</sup> Obtained from our previous papers [21, 33–35].  $K_m$  refers to  $K_m$  of pyridine N-oxide derivatives or nitroaromatic model compounds.

tends to increase with an increase in their negative  $E_{LUMO}$  values. In order to define the structure activity relationships (QSAR), the logarithm of the  $k_{cat}/K_m$  was plotted against their  $E_{LUMO}$  values. A clear correlation was obtained by the semi-empirical PM3 method ( $R = 0.95$ ,  $F = 55.85$ ), and a slightly less pronounced correlation was defined by the AM1 method ( $R = 0.92$ ,  $F = 32.27$ ). In our further studies, we have compared the reactivities of pyridine N-oxides with those of nitroaromatic model compounds. As determined in our previous work [21], the reactivity of nitroaromatics in FNR-catalyzed reactions correlated with their  $E_7^1$  values, and these reactions were analysed within the framework of the “outer-sphere” electron transfer model. This means that the reactivity of the compounds is governed by their electron accepting potency without any substantial influence of their structural peculiarities. In the present study, we have determined that the  $k_{cat}/K_m$  of nitroaromatic model compounds poorly match the reactivities of pyridine N-oxide derivatives (the resulting QSAR regressions were expressed to a less significant extent ( $R = 0.79$ ,  $F = 21.66$  and  $R = 0.78$ ,  $F = 20.61$  for AM1 and PM3 methods, respectively)). The reactivity of a series of pyridine N-oxide derivatives is generally higher than that of nitroaromatic model compounds (Fig. 2). Thus, the pyridine as well as nitropyridine N-oxide derivatives proved to be more efficient substrates for the single-electron flavin-dependent enzyme than the model nitroaromatics, and thus can be attributed to a new series of closely related compounds. In our opinion, the difference in the reactivities of pyridine N-oxide derivatives and nitroaromatic model compounds may be partly explained by differences in their self-exchange rate constants ( $k_{ex}$ ). The  $k_{ex}$  for electron transfer between the radical anion and a neutral species of a nitroaromatic compound

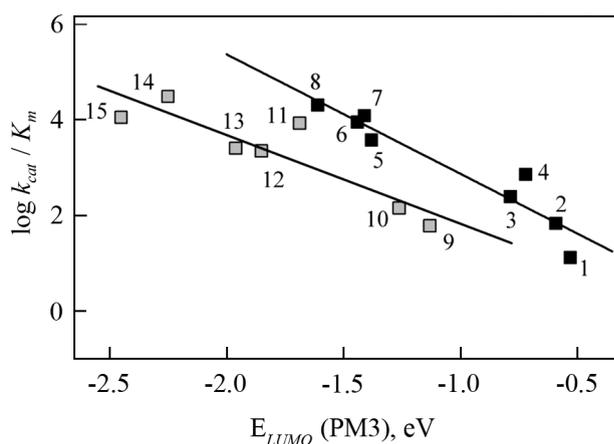


Fig. 2. Quantitative structure activity relationships describing a correlation between the logarithm of bimolecular rate constant ( $k_{cat}/K_m$ ) of reduction of pyridine N-oxide derivatives and nitroaromatic model compounds and their  $E_{LUMO}$  values.  $E_{LUMO}$  data used were those calculated by semi-empirical PM3 method. The numbers of compounds are taken from Table

is known to be  $10^4$ – $10^5 M^{-1} s^{-1}$  [31, 32]. Irrespective of the fact that the  $k_{ex}$  of pyridine N-oxides has not been determined so far, it is assumed that the higher reactivity of pyridine N-oxides can be partly caused by their  $k_{ex}$  value which seems to be higher than that of nitroaromatic model compounds.

## CONCLUSIONS

Pyridine N-oxide derivatives in the reactions with electron transferring flavoenzyme were shown to be efficient redox-active compounds. The existence of quantitative structure activity relationships for these reactions may prove to be useful

for predicting the redox activity of newly synthesized compounds of related structure and may serve as a tool for analysis of their cytotoxicity. More thorough quantum chemical and enzymatic studies for a wider range of pyridine N-oxide compounds are under way.

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## FERMENTINĖ PIRIDINO N-OKSIDO DARINIŲ REDUKCIJA: JUNGINIŲ ELEKTRONO AKCEPTORINIO PARAMETRO ĮTAKA

### Santrauka

Piridino N-oksido darinių redukcijos bei redoks ciklinimo galimybės buvo įvertintos naudojant žemo potencialo elektrontransferazinį flavininį fermentą *Anabaena* PCC 7119 ferredoksin-NADP<sup>+</sup> oksidoreduktazę (FNR, FK 1.18.1.2). Nustatyti piridino N-oksido darinių fermentinio reaktiškumo ir junginių elektrono akceptorinių parametru, apskaičiuotų kvantocheminiais metodais, struktūros-aktyvumo ryšiai (SAR). Gauti rezultatai gali būti panaudoti tolesniems tyrimams prognozuojant šių bei analogiškų junginių reaktiškumus fermentinėse reakcijose bei citotoksiškumą.