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Transient Species in the Stepwise Interconversion of NADH and NAD$^+$

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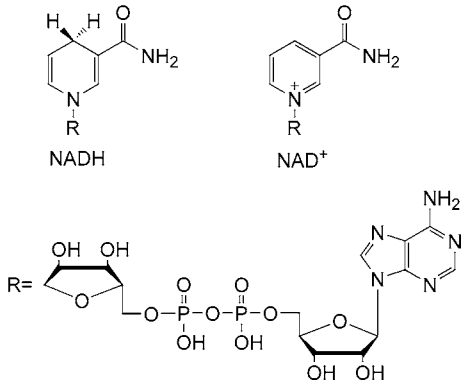
ABSTRACT
Two mechanisms of the interconversion of NADH and NAD$^+$ in the coenzyme itself and in its analogues are discussed: a one-step hydride transfer and a stepwise electron−proton−electron transfer. Direct characterization of the transient species in the stepwise process and inversion of the stability order of the keto and enol tautomers is presented. The nonenzymatic and enzyme-mediated reactions of selected pyridinium salts that affect the NADH ⇆ NAD$^+$ equilibrium are discussed in terms of their potential cytotoxicity.

Introduction

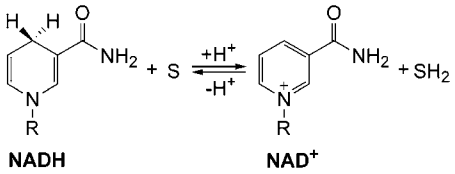
Nicotinamide adenine dinucleotide (NAD$^+$) and its reduced form (NADH), as well their 2′-phosphoric acid



derivatives, NADP$^+$ and NADPH, play a crucial role in biological systems as redox coenzymes.

In many enzymatic reactions, NAD(P)H acts as a source of two electrons and a proton. In the overall reaction, a hydride ion is transferred to a suitable substrate. In biological systems, these reversible reactions require the presence of enzymes, such as dehydrogenases. With the aid of dehydrogenases, NAD(P)H hydrogenates functional groups such as carbonyl and aldehyde groups or carbon−carbon double bonds of target substrates.[1]

Scheme 1



Over 400 enzymatic redox reactions depend on the nicotinamide coenzymes, the action of which involves the interconversion of NAD(P)H and NAD(P)$^+$. Moreover, in the mitochondria of many aerobic organisms, NADH serves as a source of two electrons to reduce molecular oxygen via the respiratory chain of reactions in which electron carriers such as flavins, quinones, and cytochromes are reduced. In addition to enzymatic reactions, the reversible interconversion of NAD(P)H and NAD(P)$^+$ is also involved in chemical reactions of NAD(P)H with hydride acceptors, such as thiobenzophenone,[2] flavins,[3] or quinones,[4] and a large group of NAD$^+$ analogues, such as acridinium or pyridinium salts.[5,6] Investigations of nonenzymatic reactions may be helpful in understanding the mechanism of the NAD(P)H ⇆ NAD(P)$^+$ interconversion, and these reactions can be of practical use, for example, in organic synthesis.[7] However, some of the nonenzymatic reactions can also have an effect on the cellular metabolism. Not only do the absolute concentrations of NADH and NAD$^+$ affect the metabolic rates, but their relative concentrations may also affect the rates of dependent reactions when the NADH/NAD$^+$ interconversion constitutes a driving force for these reactions. Cells with an impaired metabolism are more sensitive to cytotoxic agents, and therefore substrates, capable of shifting the NAD(P)H ⇆ NAD(P)$^+$ equilibrium can significantly disturb cell function and can, in extreme cases, lead to cellular death.[8]

In redox reactions, the most important role is played by the 1,4-dihydronicotinamide or the nicotinamide fragment of NAD(P)H or NAD(P)$^+$, respectively. In enzymatic or nonenzymatic reactions of nicotinamide coenzymes, a hydride anion is transferred from the 4-position of the dihydropyridine ring to the substrate (Scheme 1). The majority of mechanistic details related to the chemistry of such coenzymes were deduced from investigations of synthetic analogues containing 1,4-dihydropyridine or pyridinium cation rings, and these investigations mainly focused on the elucidation of the hydride transfer. Two possible mechanisms are discussed for this reaction: a one-step hydride ion transfer via a transition state, in which the migrating hydrogen atom carries some fractional negative charge, or a multistep hydride transfer. The latter process can occur in two steps, where an initial electron transfer is followed by the migration of a hydrogen atom, or in three steps involving an electron−proton−

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Scheme 2

One-step hydride transfer

$$NADH + A^+ \rightarrow [NAD^{\delta_{NAD^-}} \cdots H^{\delta_{H^-}} \cdots A^{\delta_{A^+}}] \rightarrow NAD^+ + HA$$

Multistep hydride transfer

electron – hydrogen atom ($e^- - H^\bullet$):

$$NADH + A^+ \rightarrow [NADH^{\bullet+} \cdots A^\bullet] \rightarrow NAD^+ + HA$$

electron – proton – electron ($e^- - H^+ - e^-$):

$$NADH + A^+ \rightarrow [NADH^{\bullet+} \cdots A^\bullet] \rightarrow [NAD^\bullet \cdots AH^{\bullet+}] \rightarrow NAD^+ + HA$$

electron transfer (Scheme 2). In the stepwise processes, radical ions or neutral radicals are formed as transient species, and their detection in the course of the reaction would constitute proof for a multistep process.

There is apparently overwhelming evidence supporting the single-step hydride transfer mechanism for both enzymatic and nonenzymatic reactions. However, the evidence against the multistep mechanism has rested largely on the lack of observation of transient species in these reactions. On this basis, arguments favoring the three- or the two-step mechanism, obtained from substituent effects, kinetic isotope effects, solvent effects, or influence of pH or oxygen on the reactions rates, were often questioned.[3,9] Moreover, the studies of hydride transfer reactions between NADH and NAD^+ analogues by Bunting et al. and Kreevoy et al. supported the conclusion that the reduction of pyridinium, quinolinium, isoquinolinium, acridinium, phenanthridinium, xanthylium, or tropylium cations (hydride acceptors) by various 1,4-dihydropyridines, 9,10-dihydroacridines, 1,4-dihydroquinolidines, or 5,6-dihydrophenanthridines (hydride donors) is a one-step process, although with a highly variable transition state structure (different fractional negative charge developing on the migrating hydrogen atom, see Scheme 2).[5,6]

If multistep hydride transfer in the interconversion of NAD(P)H and $NAD(P)^+$ is limited to examples where transient products such as radical ions or neutral radicals were detected, only few examples can be found. The explanation lies in the fact that the initial electron transfer process is usually highly endothermic and a multistep mechanism would follow if the gap of the redox potential between the NADH model compound and the reducing agent is considerably smaller than 1 V.[10] Therefore only powerful one-electron oxidants such as the ferricyanide anion, the ferrocenium cation, or inorganic or organic radicals are able to initiate a multistep process.[6,11,12]

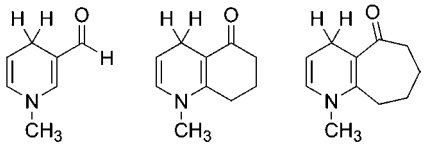
The initial electron transfer can be significantly enhanced via photoexcitation of one of the substrates[13,14] or through catalysis by acids or metal ions.[15] Intramolecular rearrangements of transient products may provide an additional driving force for electron-transfer reactions. Because the follow-up reactions of multistep conversions are often very rapid, the initial electron transfer is usually the rate-determining step in the overall process. However, the primary species formed upon electron transfer have mostly very short lifetimes, and the final products and kinetics are frequently indistinguishable for different mechanisms. These features constitute a major source of difficulties in deciding between possible interpretations of experimental data. Therefore the transient species that can participate in the NAD(P)H ⇆ $NAD(P)^+$ interconversion must be identified and their reactivity must be understood to arrive at a complete understanding of the mechanism of this reaction. These transient species may be generated selectively by radiolytic and photochemical methods, and their identification will be discussed in this Account. We will also present examples of biological systems and enzymatic reactions in which one-electron oxidation and reduction products of interest are formed.

Transient Species in the NAD(P)H ⇆ $NAD(P)^+$ Interconversion

According to the multistep mechanisms shown in Scheme 2, reactive species such as radical cations and neutral radicals, both of the hydride donor and of the acceptor molecules, are formed in the process. Such species are very reactive because they possess unpaired electrons. Localization of these electrons in different parts of a molecule may lead to the shortening or elongation of specific bonds. Determination of the spin density distribution provides information on the nature of the electronic ground state as well as on dynamic features of a species.[16] In this Account, we summarize experimental results supported by quantum mechanical calculations, which nowadays often allow spectroscopic identification and characterization of such transient species. Because experimental results directly concerning NADH and NAD^+ are scarce, most examples rest on analogues where the adenine dinucleotide residue (R in Scheme 1) is replaced by an alkyl or a benzyl group.

Radical Cations. Addition or removal of an electron significantly alters the electronic absorption (and emission) spectra of radical ions compared to their neutral precursors. Besides the electronic excitations from the occupied to the virtual orbitals that are characteristic of the parent molecules, their radical cations possess low-energy transitions within the more closely spaced occupied orbitals. In addition, the HOMO → LUMO (the highest occupied and the lowest unoccupied molecular orbital) excitation is also shifted to lower energies on removal of an electron from the HOMO, that is, on ionization.[16] In fact, radical cations generated from NADH or from its simple analogues such as *N*-methyl- or *N*-benzyl-1,4-dihydropyridines possess characteristic absorption bands in the visible region, although neutral NADH or its models absorb only at 340−360 nm.

As an example, the electronic absorption (EA) spectrum of the radical cation of *N*-methyl-3-formyl-1,4-dihydropyridine (**1H**) embedded in an argon matrix is presented



1H **2H** **3H**

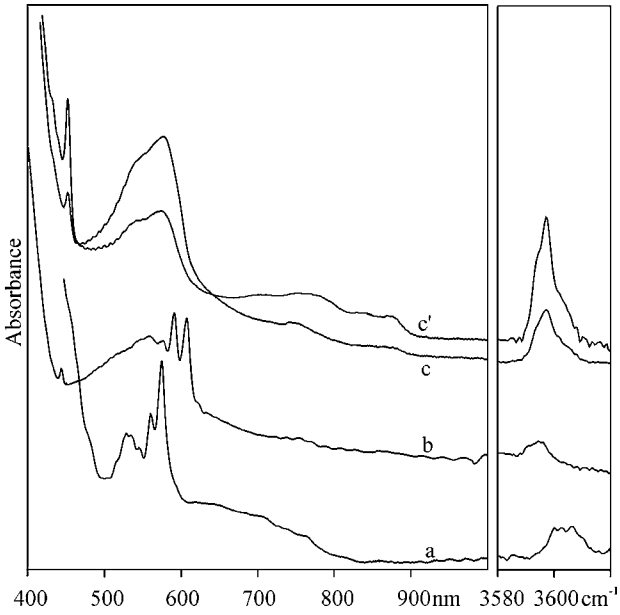


FIGURE 1. Electronic absorption and IR spectra of radical cations of **1H** (a), **2H** (b), and **3H** (c). Spectrum c′ shows spontaneous changes of spectrum c at 12 K.

in Figure 1 (spectrum a). The most prominent feature of this spectrum is a strong band with distinct vibronic fine structure located between 500 and 600 nm (λ_{max} = 575 nm). An assignment of this band to the radical cation of **1H** can be confirmed with the help of excited state calculations by the CASSCF/CASPT2 method, which has been shown to give reliable predictions in closely related cases. In addition to the lowest excitation, which carries, however, very little oscillator strength, these calculations predict an intense band of this radical cation (**1H**$^{\bullet+}$) at 575 nm (2.38 eV), in excellent agreement with the visible band in spectrum a. Analysis of the calculation results shows that this excitation corresponds mainly to electron promotion from the highest doubly occupied to the singly occupied MO (SOMO), mixed with some SOMO → LUMO excitation.[17]

A much more intense transition is predicted at 355 nm (predominantly SOMO → LUMO excitation), but it cannot be verified for this compound because it is masked by a strong absorption of the neutral precursor. However, it can be compared to the spectrum obtained by Czochralska et al. on photoionization of NADH,[13] which indeed possesses an intense band at 370 nm in addition to a much weaker one at 550 nm.

It is somewhat surprising that the spectrum of the radical cation of NADH is only observed on photoionization of the coenzyme and that it was never detected in any thermal chemical reaction. Only recently we have succeeded to identify the NADH radical cation in the reaction of NADH with one-electron oxidants such as dibromide radical anion ($Br_2^{\bullet-}$), based on the presence of the band at 500−600 nm (Figure 2A).[18]

The two characteristic absorption bands of 1,4-dihydropyridine radical cations are also predicted by the much more economical time-dependent density functional re-

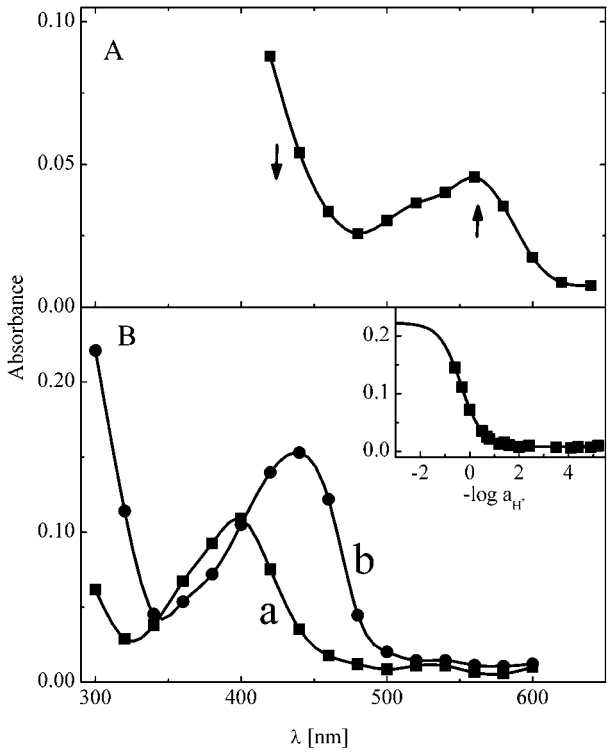
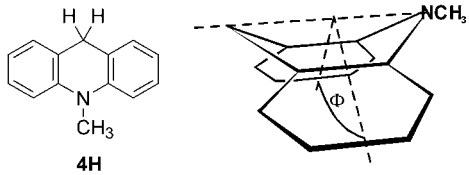


FIGURE 2. Absorption spectra of transient species generated in aqueous solution from NADH: (A) radical cation of NADH (λ_{max} = 560 nm) formed in the reaction of NADH with $Br_2^{\bullet-}$ (λ_{max} = 360 nm); (B) radical (a) and enol form of radical cation of NADH (b) obtained on reduction of NAD^+ at different pH. The inset shows the titration curve determined at 460 nm.

sponse theory (e.g., by the TD-B3LYP/6-31G* method) for other analogues of NADH.[17]

The electronic reorganization that accompanies the loss of an electron invariably entails some structural relaxation. In the case of 1,4-dihydropyridines, the geometry changes are very subtle. However, in the case of 9,10-dihydroanthracenes, geometry relaxation accompanied by the changes in the spectra can be comfortably observed.[19,20]

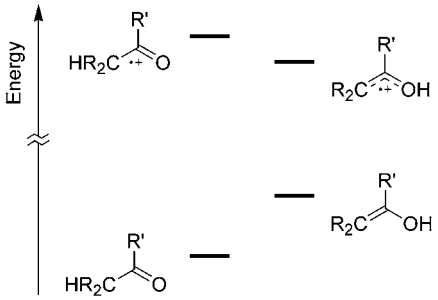
10-Methylacridan (**4H**), a compound that is frequently used to model the reactivity of NADH and its derivatives,



4H

is not planar (the B3LYP/6-31G* method predicts that the dihedral angle, Φ, between two aromatic rings is 148°). On ionization, the dihedral angle increases to 162° in **4H**$^{\bullet+}$.[20] The calculated energy for the relaxation of **4H**$^{\bullet+}$ from the neutral geometry is only 3.4 kcal/mol. This means that the potential energy surface for the relaxation is so flat that the process may be significantly inhibited in a solid matrix. Indeed, the absorption maximum of **4H**$^{\bullet+}$ is observed to gradually shift by over 50 nm (0.15 eV) on continuous softening of the matrix, as the radical cation

Scheme 3



(which is initially "frozen" in the bent geometry of the neutral precursor) relaxes to the thermodynamically more stable flattened geometry.

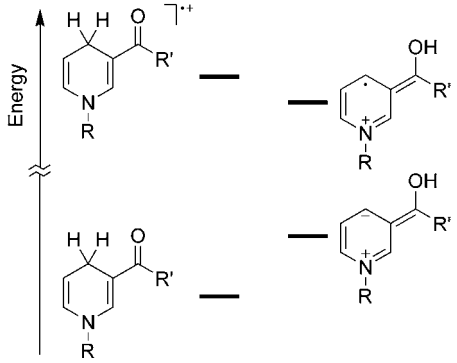
According to calculations, the structural changes on ionization of 1,4-dihydropyridines are limited to a small flattening of the puckered pyridinium ring, yet the relaxation energy remains important (a few kcal/mol).

The Enol Form of the NADH Radical Cation and Its Analogues. The inversion of the stability order of tautomeric systems on ionization is one of the unique features of molecular ions. It was calculated and found experimentally in the gas phase that such an inversion occurs for keto−enol, alkyne−allene, imine−enamine, nitrile−isonitrile, and aldimine−aminocarbene pairs.[21] Inversion of the keto−enol stability order was also observed in condensed phase (Scheme 3).[22] Enol radical cations are thermodynamically favored over their corresponding keto forms because it takes less energy to remove an electron from the π-orbital of an enol than from the n-orbital of a ketone. In addition, the structural changes of enols upon ionization, which involve a shortening of the C−O bond and an elongation of the C═C bond, are more pronounced than those in the keto-tautomers, where ionization predominantly affects the carbonyl moiety.

Due to the above-mentioned reversal of relative stabilities, it is possible under favorable circumstances to observe spontaneous enolization upon ionization of molecules embedded in matrixes. Such favorable circumstances prevail, for example, in 1,5-hydrogen transfers proceeding via six-membered ring transition states, as they occur in compounds with aromatic rings carrying an alkyl group adjacent to a carbonyl unit, for example, in *o*-carbonyl toluenes. Enolization of such compounds is strongly endothermic due to the loss of aromatic resonance energy. However, radical cations do not profit from this form of stabilization; hence, the exothermicity of their enolization is similar to that of simple ketone radical cations.[22]

According to calculations, radical cations generated from NADH models possess a thermodynamically more stable tautomer (by over 15 kcal/mol), that is, an inversion of the keto−enol stability order on ionization exists for this group of compounds (Scheme 4).[17,23] In the case of NADH radical cations, the intramolecular hydrogen atom transfer must, however, proceed via a less favorable five-membered ring transition state. Moreover, the hydrogen atoms at the 4-position of the 1,4-dihydropyridine ring lie outside the plane of the ring, whereas the accepting

Scheme 4



carbonyl group lies in that plane. This may be an important factor if the in-plane p lone pair of the oxygen atom acts as the primary acceptor of the migrating H atom.

In NADH and in 1,4-dihydropyridines with acetyl, formyl, or amide substituents at the 3-position, no spontaneous hydrogen shift is observed. The radical cations formed on ionization of these species remain in the keto form, which was characterized as mentioned earlier. Recently presented electron spin resonance (ESR) detection of radical cations formed on ionization of NADH analogues provided definitive confirmation of the keto form of these radical cations.[24]

Apparently, the process of 1,4-hydrogen shift in radical cations of NADH analogues is associated with a relatively high barrier and can only proceed efficiently if the radical cations can assume a favorable geometry. To probe the geometric requirements for efficient enolization in radical cations of NADH models, we have chosen two bicyclic derivatives: 1-methyl-1,4,7,8-tetrahydro-5(6*H*)-quinolinone (**2H**) and 1-methyl-1,4-dihydro-6,7,8,9-tetrahydro-5*H*-cyclohepta[b]pyridin-5-one (**3H**).

The spectra of the radical cations obtained on ionization of these compounds in argon matrixes show the characteristic 500−600 nm absorption bands of the keto forms (see Figure 1). However, for **3H**$^{\bullet+}$, this spectrum slowly changes, even at 12 K, into that of a new species with weak absorptions around 700−900 nm and a more intense, sharp peak at 450 nm (spectra c → c′ in Figure 1). This change is accompanied by the growth of an IR band at 3600 cm^{-1}, that is, in the region of OH stretching absorptions, typical of enol radical cations. CASSCF/CASPT2 and TD-B3LYP calculations support the assignment of the new UV−vis absorption bands to the enol radical cation.

What is the difference between **2H** and **3H** that makes that the latter radical cation undergo spontaneous tautomerization at 12 K whereas the former does not? In both compounds, the carbonyl group is incorporated into a ring and is thus locked into a syn conformation with respect to the C(4)H_2 group, which is a necessary requirement for intramolecular tautomerization. However, a proper orientation of the migrating H atom with regard to the CO group must also be secured. In the radical cations of **1H** and **2H**, the carbonyl group lies almost in the plane of

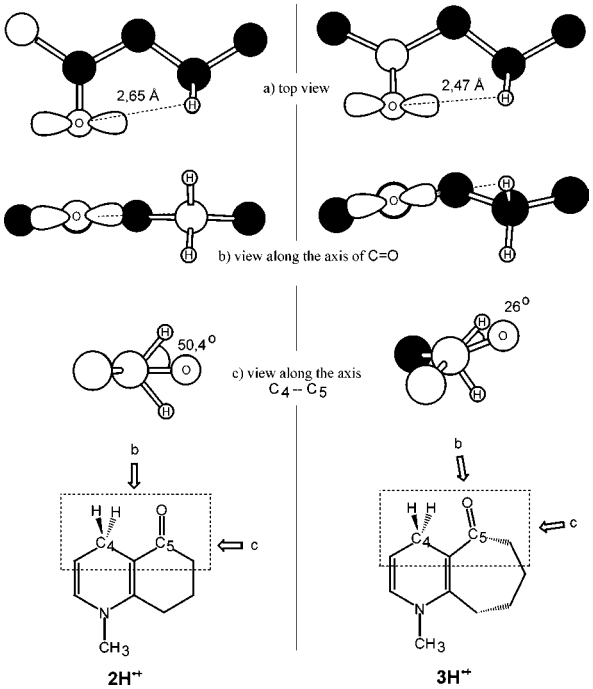


FIGURE 3. Different views of radical cations of **2H** and **3H** according to B3LYP/6-31G* calculations. Only the region boxed by the dashed line in the structural formulas is shown.

the dihydropyridine ring. However, on ionization of **3H**, the in-plane p-orbital of the C═O group aligns itself almost perfectly to accept the migrating H atom and the O···H distance shrinks from 2.65 Å in **2H**$^{\bullet+}$ to 2.47 Å in **3H**$^{\bullet+}$ (see Figure 3).[17] This clearly constitutes a more favorable geometry for the intramolecular hydrogen shift. Moreover, the structure of this radical cation mimics to some extent the syn conformation (with the amide group rotated 20°−30° out of plane) of the 1,4-dihydronicotinamide moiety of NADH observed in the enzymatically active sites.[12,25]

However, the slowness of the process compared to enolization of *o*-carbonyl toluenes[22] indicates that even the geometry of **3H**$^{\bullet+}$ is a borderline case for spontaneous enolization of radical cations generated from NADH models. Does this mean that the enol form of the NADH radical cation remains without significance in the redox processes? The answer is no, and the pertinent discussion will be presented in the following section.

Radicals. Radical cations are more acidic than their neutral precursors, and some of them are even superacids (for example, alkylated aromatics).[26] Indeed, radical cations of NADH model compounds undergo fast deprotonation in solution.[27] In some cases, the role of proton acceptor can even be played by neutral compounds. The resulting radicals can undergo rapid further decay through dimerization or oxidation. Note that oxidation of a radical is very exothermic and completes the overall process of hydride ion detachment.[28]

Radicals can, however, be stabilized and spectroscopically characterized in inert matrixes, where they can be formed through controlled deprotonation of radical cations or by one-electron reduction of closed-shell cations

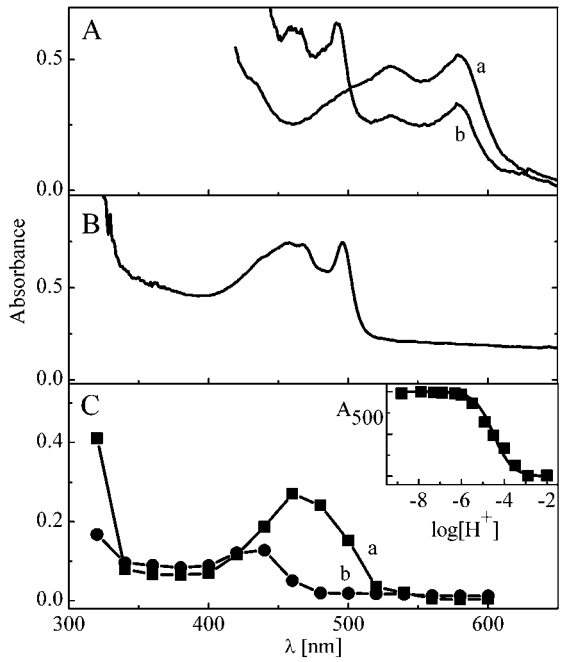
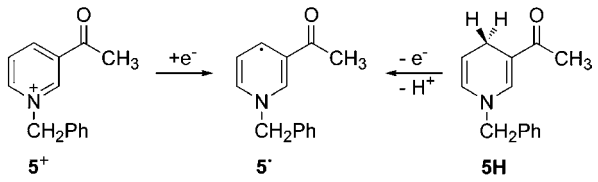


FIGURE 4. Transient absorption spectra of **5**$^{\bullet}$ obtained on (A) deprotonation of radical cation **5H**$^{\bullet+}$ (a) upon annealing of a 2-chlorobutane matrix (b), (B) reduction of **5**$^{+}$ in a 2-propanol matrix, and (C) reduction of **5**$^{+}$ in aqueous solution at pH 7 (a) and pH 3 (b). The inset shows the absorption at 500 nm vs pH.

in alcoholic matrixes.[29,30] In aqueous solution, electron addition can be monitored in pulse radiolysis experiments.[31]

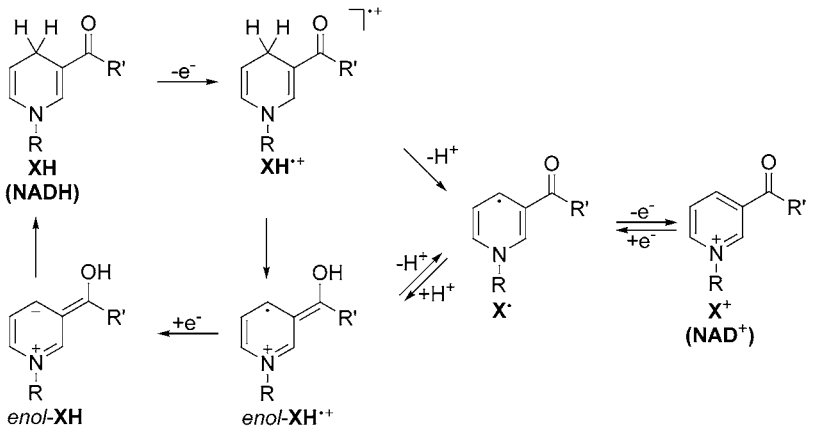
The spectra of the radicals obtained by one-electron oxidation of *N*-benzyl-3-acetyl-1,4-dihydropyridine (**5H**)



or by one-electron reduction of its oxidized form (**5**$^{+}$) are presented in Figure 4. The most prominent feature of these spectra is a band located at 400−500 nm. Under matrix conditions, it shows some vibronic structure, but it remains structureless in solution. CASSCF/CASPT2 calculations show that this band corresponds to the second excited state (the lowest energy HOMO → LUMO excitation in near-IR cannot be detected because it has a very small oscillator strength). A much stronger band predicted below 300 nm agrees well with the observed strong increase in the absorption of the radical below 320 nm.

Under acidic conditions, the radical is unstable and it is protonated so rapidly that the rate constant of the process can only be estimated as $>10^{10}$ M^{-1} s^{-1}. Protonation of the radical leads to the radical cation, and since the keto radical cation (obtained by protonation at C-4) is less stable than the corresponding enol form (obtained by O-protonation), it is reasonable to assume that the latter is being formed predominantly. Indeed, an absorp-

Scheme 5



tion band of this new species lies at 400−450 nm (spectrum b in Figure 4c), and it agrees well with the sharp band observed at 450 nm for the enol radical cation of **3H**$^{\bullet+}$, which is formed by spontaneous intramolecular proton transfer (spectrum c′ in Figure 1).

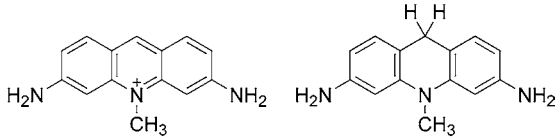
Monitoring the absorption of the radical at 500 nm as a function of pH gave the titration curve shown as an inset to Figure 4C. From the titration curves, we can deduce pK_a values of 4.3−4.5 for compounds carrying formyl or acetyl substituents in the 3-position.[29] For NADH analogues carrying an amide group, these values are about three units lower. We were also able to identify the enol form of the radical cation of NADH itself, but its formation from the NAD$^{\bullet}$ radical requires extremely acidic conditions (pK_a < 0, see inset in Figure 2B).[18]

Although intramolecular tautomerization of NADH analogues depends strongly on the geometry of the molecule and can therefore not always be achieved easily, this process may be assisted (or catalyzed) by the neutral parent compound acting as a proton shuttle. Calculations show that it is energetically feasible for the keto radical cation to transfer a proton to a neutral molecule, which in turn protonates a neutral radical to give the enol radical cation.[29,30,32] Moreover, there are examples of direct enol radical cation formation upon photoionization of NADH analogues.[24,30]

The cycle of reactions involved in the NAD(P)H ⇄ NAD(P)$^+$ interconversion is summarized in Scheme 5. Note, however, that the removal of an electron from NADH or its analogues initiates subsequent proton and electron transfers, whereas the reverse process, initiated by addition of an electron to the closed-shell cation NAD$^+$, cannot be easily accomplished. One-electron reduction of NAD$^+$ analogues does not lead to an overall hydride ion addition in the same way that the one-electron oxidation of NADH triggers multistep hydride anion detachment.

Transient Species in Biological Systems

Two examples that show the importance of transient species formed on oxidation and reduction of analogues of NADH and NAD$^+$, respectively, in biological systems will be presented in this section. Acriflavine (**6**$^+$) represents



a group of cationic DNA intercalators that possess outstanding antimicrobial properties, despite the fact that only a very small fraction of the molecules can penetrate inside bacterial cells to interact with the genetic material, mainly because of their charge.[33] On the other hand, its reduced form, 3,6-diamino-10-methylacridan (**6H**), is not an efficient DNA intercalator because it is not planar.[20] However, being an uncharged, hydrophobic acriflavine precursor it readily penetrates cell membranes. It was in fact found that **6H** possesses much better antimicrobial properties (against bacteria, fungi, and viruses) than acriflavine itself.[34] It was also shown that **6H** can be quantitatively oxidized to **6**$^+$ by molecular oxygen and therefore serve as a source of both acriflavine and superoxide radical anion ($O_2^{\bullet-}$). Upon dissolution of **6H** in aerated water or other polar solvents, spontaneous oxidation takes place (see Figure 5). Because of the relatively low oxidation potential of **6H**, its oxidation to **6**$^+$ may involve a sequential electron−proton−electron transfer. The superoxide radical anion that is formed simultaneously also has pronounced antimicrobial properties. Over 99% of **6H** reacts with molecular oxygen by this route, while less than 1% leads to 3,6-diamino-10-methylacridan-9-one, another oxidation product.

The above example shows that easily oxidized analogues of NADH (the 1,4-dihydropyridine ring is a central fragment of **6H**) carry some potential as antimicrobial agents.

As mentioned in the Introduction, pyridinium salts can react reversibly with NADH by hydride anion transfer. Introducing an agent capable of shifting the NAD(P)H ⇆ NAD(P)$^+$ equilibrium in a cell can disturb the cell function, which may be lethal for cells with impaired metabolism. Strategies to control cell growth by affecting the NADH level and ensuing adenosine triphosphate depletion have already been presented in the literature.[8] Investigations of a group of 1-methylpyridinium salts carrying various

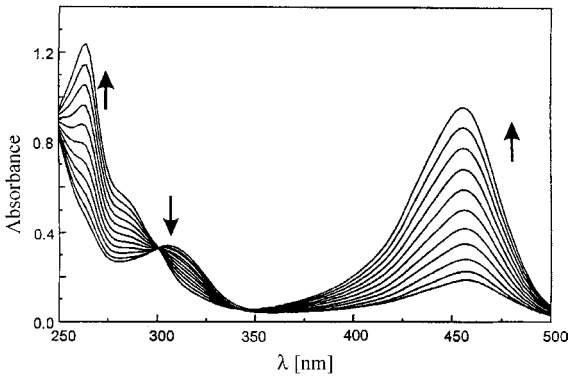
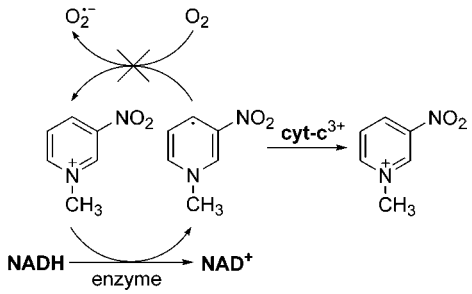
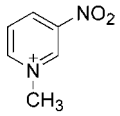


FIGURE 5. Oxidation of **6H** (λ_{max} = 302 nm) into $\mathbf{6}^+$ (λ_{max} = 263 and 454 nm) in air-saturated aqueous solution.

Scheme 6



substituents at the 3-position to tune their redox properties have indeed revealed cytotoxic properties of these compounds in murine leukemia L1210 cell cultures. ED_{50} values (the drug concentration effective in inhibiting 50% of the cell growth after 72 h exposure of L1210 cells to the drug) show a correlation with the redox properties for this group of compounds.[35] A surprisingly strong effect is observed for the 1-methyl-3-nitropyridinium cation ($\mathbf{7}^+$).[36]



$\mathbf{7}^+$

Similarly to other compounds, $\mathbf{7}^+$ can effectively oxidize NADH in a nonenzymatic process, which probably involves a one-step hydride transfer because no intermediates were detected in the course of the NADH oxidation. However, in contrast to the other pyridinium salts, $\mathbf{7}^+$ can also effectively oxidize NADH in an enzyme-mediated process.

It is known that NADH can be a substrate for a number of flavoenzymes, for example, for xanthine oxidase (XO).[37] XO-mediated oxidation of NADH to NAD^+ results also in the formation of superoxide radical anion ($O_2^{\bullet-}$). This process can be activated by a number of easily reducible agents, which catalyze formation of $O_2^{\bullet-}$.[37] It was found that $\mathbf{7}^+$ can also activate oxidation of NADH mediated by XO.

NADH interacting with the flavin center of XO causes its reduction, and $\mathbf{7}^+$ reoxidizes the enzyme (Scheme 6). The formation of the radical $\mathbf{7}^\bullet$ in this reaction is confirmed by the one-electron reduction of cytochrome *c* (cyt c^{3+}), which occurs at the same rate as the NADH oxidation and is not affected by oxygen. The experiments conducted in the presence of superoxide dismutase (SOD) exclude formation of $O_2^{\bullet-}$ in the enzymatic reaction. Also, pulse radiolysis experiments show no reaction of $\mathbf{7}^\bullet$ with oxygen that is faster than its reaction with cyt c^{3+} ($k = 1.9 \times 10^6$ M^{-1} s^{-1}).

On the basis of the evidence presented above that $\mathbf{7}^+$ can effectively oxidize NADH in nonenzymatic or enzyme-mediated processes with formation of radicals, one can expect that $\mathbf{7}^+$ can also function as the cytotoxic agent. Indeed it was found that $\mathbf{7}^+$ possesses remarkable cytotoxic activity against cultured murine leukemia L1210 cells. In terms of ED_{50}, $\mathbf{7}^+$ was found to be a very active antiproliferative agent ($ED_{50} = 3\ \mu M$) with activity comparable to that of the antitumor drug cisplatin.[38] It is conceivable that formation of radicals in the enzyme-mediated processes activated by $\mathbf{7}^+$ is mostly responsible for the cytotoxicity observed.

The two examples presented above show the importance of the multistep NAD(P)H ⇆ $NAD(P)^+$ interconversion and the important role of the species generated in this process. A significant pharmacological potential may arise from the participation of the transient species generated in these processes.

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References

(1) Stryer, L. *Biochemistry*; Freeman: New York, 1995.
(2) Abeles, R. H.; Hutton, R. F.; Westheimer, F. H. The Reduction of Thioketones by a Model for a Coenzyme. *J. Am. Chem. Soc.* **1957**, *79*, 712−716.
(3) Powell, M. F.; Bruice, T. C. Hydride vs Electron Transfer in the Reduction of Flavin and Flavin Radical by 1,4-Dihydropyridines. *J. Am. Chem. Soc.* **1983**, *105*, 1014−1021.
(4) Carlson, B. W.; Miller, L. L. Mechanism of the Oxidation of NADH by Quinones. Energetics of One-Electron and Hydride Routes. *J. Am. Chem. Soc.* **1985**, *107*, 479−485.
(5) (a) Ostovič, D.; Lee, I. S. H.; Roberts, R. M. G.; Kreevoy, M. M. Hydride Transfer and Oxyanion Addition Equilibria of NAD^+ Analogues. *J. Org. Chem.* **1985**, *50*, 4206−4211. (b) Kreevoy, M. M.; Ostovič, D.; Lee, I. S. H.; Binder, D. A.; King, G. W. Structure Sensitivity of the Marcus λ for Hydride Transfer between NAD^+ Analogues. *J. Am. Chem. Soc.* **1988**, *110*, 524−530. (c) Lee, I.-S. H.; Jeoung, E. H.; Kreevoy, M. M. Marcus Theory of a Parallel Effect on α for Hydride Transfer Reaction between NAD^+ Analogues. *J. Am. Chem. Soc.* **1997**, *119*, 2722−2728.
(6) Bunting, J. Merged Mechanisms for Hydride Transfer from 1,4-Dihydronicotinamide. *Bioorg. Chem.* **1991**, *19*, 456−491.
(7) Taylor, K. E.; Jones, J. B. Nicotinamide Coenzyme Regeneration by Dihydropyridine and Pyridinium Compounds. *J. Am. Chem. Soc.* **1976**, *98*, 5689−5694.
(8) (a) Morton, R. K. Enzymic Synthesis of Coenzyme I in Relation to Chemical Control of Cell Growth. *Nature* **1958**, *181*, 540−542. (b) Boulton, S.; Kyle, S.; Durkacz, B. W. Low Nicotinamide Mononucleotide Adenylyltransferase Activity in a Tiazofurin-resistant Cell Line: Effects on NAD Metabolism and DNA Repair. *Br. J. Cancer* **1997**, *76*, 845−851.
(9) Powell, M. F.; Bruice, T. C. Effect of Isotope Scrambling and Tunneling on the Kinetic and Product Isotope Effects for Reduced Nicotinamide Adenine Dinucleotide Model Hydride Transfer Reactions. *J. Am. Chem. Soc.* **1983**, *105*, 7139−7149.

(10) Cheng, J.-P.; Lu, Y.; Zhu, X.; Mu, L. Energetics of Multistep versus One-step Hydride Transfer Reactions of Reduced Nicotinamide Adenine Dinucleotide (NADH) Models with Organic Cations and p-Quinones. *J. Org. Chem.* **1998**, *63*, 6108−6114.
(11) (a) Grodkowski, J.; Neta, P.; Carlson, B. W.; Miller, L. L. One-Electron-Transfer Reactions of the Couple NAD•/NADH. *J. Phys. Chem.* **1983**, *87*, 3135−3138. (b) Carlson, B. W.; Miller, L. L.; Neta, P.; Grodkowski, J. Oxidation of NADH Involving Rate-Limiting One-Electron Transfer. *J. Am. Chem. Soc.* **1984**, *106*, 7233−7239. (c) Powell, M. F.; Wu, J. C.; Bruice, T. C. Ferricyanide Oxidation of Dihydropyridines and Analogues. *J. Am. Chem. Soc.* **1984**, *106*, 3850−3856. (d) Miller, L. L.; Valentine, J. R. On the Electron-Proton-Electron Mechanism for 1-Benzyl-1,4-Dihydronicotinamide Oxidations. *J. Am. Chem. Soc.* **1988**, *110*, 3982−3989. (e) Brewster, M. E.; Kaminski, J. J.; Gabanyi, Z.; Czako, K.; Simay, A.; Bodor, N. Reactivity of Biologically Important Reduced Pyridines VI. Lack of Through-Resonance Stabilization in the Ferricyanide-mediated Oxidation of Substituted 1-Phenyl-1,4-Dihydronicotinamides. *Tetrahedron* **1990**, *46*, 319−324.
(12) Almarsson, Ö.; Sinha, A.; Gopinath, E.; Bruice, T. C. Mechanism of One-Electron Oxidation of NAD(P)H and Function of NADPH Bound to Catalase. *J. Am. Chem. Soc.* **1993**, *115*, 7093−7102.
(13) Czochralska, B.; Lindqvist, L. Biphotonic One-Electron Oxidation of NADH on Laser Excitation at 353 nm. *Chem. Phys. Lett.* **1983**, *101*, 297−299.
(14) Fukuzumi, S.; Tanaka, T. NAD(P)H, NAD^+, and Analogues. In *Photoinduced Electron Transfer*; Fox, M. A., Chanon, M., Eds.; Elsevier: Amsterdam, 1988; Part C., pp 578−635.
(15) Fukuzumi, S. Catalysis on Electron Transfer and the Mechanistic Insight into Redox Reactions. *Bull. Chem. Soc. Jpn.* **1997**, *70*, 1−28.
(16) Gębicki, J.; Marcinek, A. Radical Ions: Generation, Characterization and Reactions. In *General Aspects of the Chemistry of Radicals*; Alfassi, Z. B., Ed.; Wiley: Chichester, U.K., 1999; pp 175−208.
(17) Marcinek, A.; Adamus, J.; Huben, K.; Gębicki, J.; Bartczak, T. J.; Bednarek, P.; Bally, T. Hydrogen-Transferred Radical Cations of NADH Model Compounds. 1. Spontaneous Tautomerization. *J. Am. Chem. Soc.* **2000**, *122*, 437−443.
(18) Zielonka, J.; Marcinek, A.; Adamus, J.; Gębicki, J. Direct Observation of NADH Radical Cation Generated in Reactions with One-electron Oxidants. *J. Phys. Chem. A* **2003**, *107*, 9860−9864.
(19) Marcinek, A.; Rogowski, J.; Adamus, J.; Gębicki, J.; Platz, M. S. Sequential Electron−Proton-Electron Transfer in the Radiolytic Oxidation of Thioxanthene and Xanthene. *J. Phys. Chem.* **1996**, *100*, 13539−13543.
(20) Marcinek, A.; Zielonka, J.; Adamus, J.; Gębicki, J.; Platz, M. S. Direct Characterization of Radical Species Generated on One-Electron Oxidation of 3,6-Diamino-10-methylacridan. *J. Phys. Chem. A* **2001**, *105*, 875−879.
(21) Schmittel, M. Umpolung of Ketones via Enol Radical Cations. *Top. Curr. Chem.* **1994**, *169*, 183−230.
(22) (a) Marcinek, A.; Michalak, J.; Rogowski, J.; Tang, W.; Bally, T.; Gębicki, J. Enolization in Radical Cations of o-Methylacetophenone and Related Species under Cryogenic Conditions. *J. Chem. Soc., Perkin Trans. 2* **1992**, 1353−1357. (b) Gębicki, J.; Bally, T. Spontaneous Hydrogen Atom Transfer on Ionization: Characterization of Enol Radical Cations in Cryogenic Matrices. *Acc. Chem. Res.* **1997**, *30*, 477−485.
(23) Gębicki, J.; Marcinek, A.; Adamus, J.; Paneth, P.; Rogowski, J. Structural Aspects and Rearrangement of Radical Cations Generated from NADH Analogues. *J. Am. Chem. Soc.* **1996**, *118*, 691−692.
(24) Fukuzumi, S.; Inada, O.; Suenobu, T. Mechanisms of Electron-Transfer Oxidation of NADH Analogues and Chemiluminescence. Detection of the Keto and Enol Radical Cations. *J. Am. Chem. Soc.* **2003**, *125*, 4808−4816.
(25) (a) Oppenheimer, N. J.; Handlon, A. L. Mechanism of NAD-Dependent Enzymes. In *The Enzymes*; Sigman, D. S., Ed.; Academic Press: San Diego, CA, 1992; Vol. 20, pp 453−505. (b) Wu, Y.-D.; Houk, K. N. Theoretical Study of Conformational Features of NAD^+ and NADH Analogues: Protonated Nicotinamide and 1,4-Dihydronicotinamide. *J. Org. Chem.* **1993**, *58*, 2043−2045.
(26) Bordwell, F. G.; Cheng, J.-P. Radical-Cation Acidities in Solution and in the Gas Phase. *J. Am. Chem. Soc.* **1989**, *111*, 1792−1795.
(27) (a) Anne, A.; Hapiot, P.; Moiroux, J.; Neta, P.; Savéant, J.-M. Oxidation of 10-Methylacridan, a Synthetic Analogue of NADH, and Deprotonation of Its Cation Radical. Convergent Application of Laser Flash Photolysis and Direct and Redox Catalyzed Electrochemistry to the Kinetics of Deprotonation of the Cation Radical. *J. Phys. Chem.* **1991**, *95*, 2370−2377. (b) Anne, A.; Hapiot, P.; Moiroux, J.; Neta, P.; Savéant, J.-M. Dynamics of Proton Transfer from Cation Radicals. Kinetic and Thermodynamic Acidities of Cation Radicals of NADH Analogues. *J. Am. Chem. Soc.* **1992**, *114*, 4694−4701.
(28) (a) Land, E. J.; Swallow, A. J. One-Electron Reactions in Biochemical Systems as Studied by Pulse Radiolysis. IV. Oxidation of Dihydronicotinamide-Adenine Dinucleotide. *Biochim. Biophys. Acta* **1971**, *234*, 34−42. (b) Moiroux, J.; Elving, P. J. Mechanistic Aspects of the Electrochemical Oxidation of Dihydronicotinamide Adenine Dinucleotide (NADH). *J. Am. Chem. Soc.* **1980**, *102*, 6533−6538. (c) Hapiot, P.; Moiroux, J.; Savéant, J.-M. Electrochemistry of NADH/NAD^+ Analogues. A Detailed Mechanistic Kinetic and Thermodynamic Analysis of the 10-Methylacridan/10-Methylacridinium Couple in Acetonitrile. *J. Am. Chem. Soc.* **1990**, *112*, 1337−1343. (d) Adamus, J.; Rogowski, J.; Michalak, J.; Paneth, P.; Gębicki, J.; Marcinek, A.; Platz, M. S. Photochemical and Radiolytic Cleavage of 10-Methylacridine Dimer in Solution and Cryogenic Glasses. *J. Phys. Org. Chem.* **1993**, *6*, 254−256.
(29) Marcinek, A.; Rogowski, J.; Adamus, J.; Gębicki, J.; Bednarek, P.; Bally, T. Hydrogen-Transferred Radical Cations of NADH Model Compounds. 2. Sequential Electron−Proton Addition to NAD^+. *J. Phys. Chem. A* **2000**, *104*, 718−723.
(30) Marcinek, A.; Adamus, J.; Gębicki, J.; Platz, M. S.; Bednarek, P. Hydrogen-Transferred Radical Cations of NADH Model Compounds. 3. 1,8-Acridinediones. *J. Phys. Chem. A* **2000**, *104*, 724−728.
(31) (a) Land, E. J.; Swallow, A. J. One-Electron Reactions in Biochemical Systems as Studied by Pulse Radiolysis. I. Nicotinamide-Adenine Dinucleotide and Related Compounds. *Biochim. Biophys. Acta* **1968**, *162*, 327−337. (b) Brühlmann, U.; Hayon, E. One-Electron Redox Reactions of Water-Soluble Vitamins. I. Nicotinamide (Vitamin B_5) and Related Compounds. *J. Am. Chem. Soc.* **1974**, *96*, 6169−6175. (c) Neta, P.; Patterson, L. K. Substituted Pyridinyl Radicals in Aqueous Solutions. Formation, Reactivity, and Acid−Base Equilibria. *J. Phys. Chem.* **1974**, *78*, 2211−2217. (d) Kosower, E. M.; Teuerstein, A.; Burrows, H. D.; Swallow, A. J. Bimolecular Reactions of Pyridinyl Radicals in Water and the Mechanism of NAD^+-NADH Dehydrogenase Reactions. *J. Am. Chem. Soc.* **1978**, *100*, 5185−5190.
(32) Marcinek, A.; Zielonka, J.; Gębicki, J.; Gordon, C. M.; Dunkin, I. R. Ionic Liquids: Novel Media for Characterization of Radical Ions. *J. Phys. Chem. A* **2001**, *105*, 9305−9309.
(33) (a) Dean, A. C. R. The Antibacterial Action of Acridines. In *Acridines*, 2nd ed.; Acheson, R. M., Ed.; Interscience Publishers: New York, 1973; pp 789−813. (b) Nikaido, H. Prevention of Drug Access to Bacterial Targets: Permeability Barriers and Active Efflux. *Science* **1994**, *264*, 382−388.
(34) Adamus, J.; Gębicki, J.; Ciebiada, I.; Korczak, E.; Denys, A. 3,6-Diamino-10-methylacridan: Uncharged Precursor of Acriflavine and Its Unique Antimicrobial Activity. *J. Med. Chem.* **1998**, *41*, 2932−2933.
(35) Wieczorkowska, M.; Adamnus, J.; Zielonka, J.; Marcinck, A.; Gębicki, J., manuscript in preparation.
(36) Wieczorkowska, M.; Zielonka, J.; Celińska, J.; Adamus, J.; Ciesielska, E.; Marcinek, A.; Szmigiero, L.; Gębicki, J. 1-Methyl-3-nitropyridine: An Efficient Oxidant of NADH in Nonenzymatic and Enzyme-mediated Processes. *Free Radical Res.* **2003**, *37*, 1157−1162.
(37) Harrison, R. Xanthine oxidoreductase. In *Free Radicals and Inflammation*; Winyard, P. G., Blake, D. R., Evans, C. H., Eds.; Birkhäuser: Basel, Switzerland, 2000; pp 65−81.
(38) Ciesielska, E.; Studzian, K.; Zyner, E.; Ochocki, J.; Szmigiero, L. DNA Damage and Apoptosis Induction in L1210 Cells by Cis-Diaminedichloroplatinum(II) and Its New Aminoflavone Analogue. *Cell. Mol. Biol. Lett.* **2000**, *5*, 441−450.

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