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Expanding Reaction Horizons: Evidence of the 5-Deazaflavin Radical Through Photochemically Induced Dynamic Nuclear Polarization

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Dedicated to Professor Silvia Braslavsky on the occasion of her 80th birthday

Abstract: Deazaflavins are important analogues of the naturally occurring flavins: riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD). The use of 5-deazaflavin as a replacement coenzyme in a number of flavoproteins has proven particularly valuable in unraveling and manipulating their reaction mechanisms. It was frequently reported that one-electron-transfer reactions in flavoproteins are impeded with 5-deazaflavin as the cofactor. Based on these findings, it was concluded that the 5-deazaflavin radical is significantly less stable compared to the respective flavin semiquinone and quickly re-oxidizes or undergoes disproportionation. The long-standing paradigm of 5-deazaflavin being solely a two-electron/hydride acceptor/donor—"a nicotinamide in flavin clothing"—needs to be re-evaluated now with the indirect observation of a one-electron-reduced (paramagnetic) species using photochemically induced dynamic nuclear polarization (photo-CIDNP) ^{1}H nuclear magnetic resonance (NMR) under biologically relevant conditions.

Introduction

5-Deazaflavin and its derivatives are frequently used as replacement coenzymes of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) when it comes to unraveling and altering the reaction mechanisms of flavoproteins; early work has been reviewed by Hemmerich, Massey and Fenner.[1] With a 5-deazaflavin cofactor, one modification—the N5 of the flavins' 7,8-dimethyl isoalloxazine moiety is replaced by C5-H in 5-deazaflavins, see Figure 1—has tremendous effect on the electrochemical and photochemical properties of a protein while largely preserving a cofactor binding situation similar to the one of the respective flavin chromophore it has replaced. Based on the fact that one-electron-transfer reactions in flavoproteins with 5-deazaflavin as cofactor are frequently impeded, it was concluded that 5-deazaflavin radicals are significantly less stable as compared to the respective flavin semiquinones, and quickly re-oxidize or undergo disproportionation.[1–4] The stability of the fully (two-electron) reduced form of 5-deazaflavin towards oxygen favors the formation of dimerization products.[5–6] Hence, when bound to electron-transfer competent enzymes, 5-deazaflavins act as two-electron (hydride) acceptors/donors rather than as one-electron acceptors/donors.[7–8]

Two 5-deazaflavin derivatives, named F_{420} and F_o (the latter is also sometimes referred to as 8-HDF (8-hydroxy-5-deazaflavin) for 7,8-didemethyl-8-hydroxy-5-deazariboflavin, F_0 or F_O, see Figure 1) are known to act as cofactors,[9–12] e.g., in the DNA-repair enzymes DNA photolyase of the 8-HDF type as the second, light-harvesting chromophore[13–14] that significantly accelerates the reaction rate in low-light conditions. Also the blue-light sensitivity of *Cr*aCRY, a cryptochrome from the green alga *Chlamydomonas reinhardtii*, is enhanced by the protein's capability to incorporate 8-HDF as antenna chromophore.[15] The 5-deazaflavin F_{420} is required, e.g., in methanogenesis (i.e., the anaerobic production of methane from CO_2 or simple carbon compounds).[16]

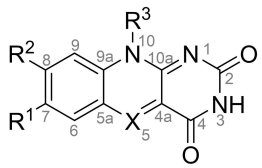

Figure 1. Structure of riboflavin and 5-deazaflavin analogs: R^1=R^2=Me, R^3 = ribityl 5′-phosphate, X=N: FMN; R^1=R^2=Me, R^3 = ribityl 5′-phosphate, X=CH: 5-deazaFMN; R^1=H, R^2=OH, R^3 = ribityl 5′-phospho-oligoglutamate, X=CH: F_{420}; R^1=H, R^2=OH, R^3 = ribityl, X=CH: F_o.

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The F_{420} core, 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5′-phosphate, is also involved in archaea and in Gram-positive eubacteria, such as *Streptomyces*[11] or *Mycobacteria*.[17]

5-Deazaflavin derivatives were recently identified by biochemical screening for inhibitors of tyrosyl-DNA phosphodiesterase 2 (TDP2),[18–19] a DNA-repair enzyme for irreversible topoisomerase-II-mediated cleavage complexes. Due to the stability of their fully reduced states towards oxygen, 5-deazaflavins are also potentially useful photocatalysts for the direct, nicotinamide-independent regeneration of flavoenzymes.[20]

Very recently, derivatized 5-deazaflavins were synthesized and introduced as potent photocatalysts with a reductive power comparable to that of sodium and lithium.[21–22] By choosing Br or Cl as substituent, e.g., at position R^1 (see Figure 1), the triplet yield was maximized and the value of such tuned "photocatalysts of the next generation" demonstrated in light-induced dehalogenation of arenes that proceeds via a one-electron reduced deazaflavin species.[22]

Two remarkable examples of how a protein's behavior may be modified by simply replacing the native flavin cofactor by the respective 5-deazaflavin are, firstly, the recently demonstrated conversion of a dehalogenase into a nitrogenase[23] and, secondly, the dramatic enhancement of the photoswitch capability of the blue-light photoreceptor domain of phot1, LOV2. The latter studies were conducted in our laboratories,[24] and later extended by Gärtner and co-workers to the related YtvA protein from *Bacillus subtilis*,[25] which is involved in the light-regulated stress response.

As cofactor, LOV domains harbor FMN in its fully oxidized redox state absorbing at around 445 nm; for recent reviews, see refs. [26–27]. Absorption of blue light initiates a photocycle that proceeds via the flavin's triplet state to the signaling state:[28] a photoadduct, in which the flavin forms a covalent bond to a neighboring cysteine residue via its C4a atom.[29–31] This photoadduct absorbs at 390 nm. Recovery of the ground state is thermally driven; a large variety in photoadduct lifetimes ranging from several seconds in the *Arabidopsis thaliana* LOV proteins phot1 and phot2,[32–33] to more than 10,000 seconds in *Neurospora crassa* VIVID[34] is observed. By replacing FMN by 5-deazaFMN, a similar photocycle may be initiated, albeit with light of lower wavelengths, due to the 5-deazaflavin's blue-shifted absorbance ($\lambda_{max} = 405$ nm). The generated signaling state, however, is very long-lived (for several days) as compared to the wild-type protein carrying FMN.[24–25] It may be photoswitched back to the parent state by UV-A light (340 nm) although not completely because 5-deazaFMN and the photoadduct have overlapping absorbance. This switching cycle is very robust and may be repeated dozens of times with negligible loss in efficiency.

From the magnetic resonance point of view, very little information is available on the radical form of 5-deazaflavin and its derivatives. Hersh and co-workers reported in 1977 a continuous-wave (cw) electron paramagnetic resonance (EPR) signature supposedly of a 5-deazaFAD radical bound to D-amino acid oxidase from pig kidney.[35] The signal was poorly resolved and rather noisy. A spectral simulation was not attempted by the authors. The lack of independent data from other types of EPR, such as electron-nuclear double resonance (ENDOR),[36] electron-spin echo envelope modulation (ESEEM),[37] and hyperfine sublevel correlation (HYSCORE),[37] by which hyperfine couplings[38] can be detected, would have made a meaningful simulation questionable anyway.

From the aforementioned 5-deazaflavin-based photocatalysts, a radical derived from the C5-phenylated derivative (with further modifications: $R^1{=}R^2{=}OCH_3$, N3-CH_3 instead of N3-H, and N10-CH_3 instead of N10-ribityl 5′-phosphate) was characterized using cw EPR.[21] Three hyperfine couplings of substantial strength could be identified. These were tentatively assigned to N10 and the 2′ and 6′ protons of the position 5 phenyl substituent. Sizeable proton hyperfine couplings from the phenyl substituent point to efficient delocalization of the unpaired electron spin beyond the aromatic 5-deazaflavin core, which may promote stabilization of the one-electron reduced species.

Driven by the lack of knowledge of the unsubstituted 5-deazaflavin radical and motivated by a recent report on the catalytic capabilities of C5-derivatized 5-deazaflavins[21] we applied photo-CIDNP, an NMR technique frequently used to study cyclic chemical photoreactions involving short-lived radicals.[39–41] This method often reveals elusive paramagnetic species that even escape detection by EPR.[39,42–45] Photo-CIDNP in solution relies on the fact that a radical pair state generated by photo-induced electron transfer is formed with a multiplicity that is equal to that of its precursor state due to the conservation of spin angular momentum. This could be an (excited) singlet state (spin quantum number $S = 0$) or, if intersystem crossing precedes electron transfer, a triplet state ($S = 1$). In the latter case, a triplet-configured radical pair is generated. Immediately after the generation of the radical pair, two phenomena occur simultaneously:[40–41,46] (i) The multiplicity of the radical pair oscillates between singlet and triplet with a frequency that depends, apart from the dipolar and exchange couplings between the radicals, on differences in the g values and hyperfine couplings of the radical pair halves. (ii) In the liquid state the radicals may diffuse with the possibility of recombining after a possible reencounter. Since only singlet-configured radical pairs are allowed to directly recombine to diamagnetic products upon such an encounter, triplet-configured radical pairs tend to separate and eventually form so-called (diamagnetic) "escape products". Both processes lead to spin sorting; in the end each reaction product favors a certain nuclear spin configuration, which leads to hyperpolarized NMR resonances of the diamagnetic products.[40,47] Analysis of NMR hyperpolarization yields information on the precursor state and on the hyperfine structure of the radicals involved in the photochemical process under consideration.[48] In the time-resolved variant of photo-CIDNP, tr-photo-CIDNP, the NMR signal amplitudes are proportional to the isotropic hyperfine coupling constant of the respective nucleus.[39]

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Results and Discussion

We have conducted ^{1}H NMR experiments with aqueous solutions of 5-deazaFMN (0.2 mM) at various pH values. The assignment of ^{1}H resonances of the redox-active moiety of 5-deazaFMN was achieved by analyzing NOESY data (see Figure S1 in the Supporting Information). Signals at around 2.25 and 2.4 ppm arise from the methyl protons H7α and H8α, respectively. In the aromatic range, resonances from H6, H9 and H5 are found at around 7.6, 7.7 and 8.6 ppm, respectively. The pH dependence of the ^{1}H chemical shifts over a wide range $1 < pH < 12.5$ is shown in Figure S2.

Tr-photo-CIDNP ^{1}H NMR experiments were performed with aqueous 5-deazaFMN (0.2 mM) solutions to which a 25-fold excess of L-tryptophan was added. Light excitation (420 nm) was achieved by coupling the output of a nanosecond-pulsed Nd:YAG/OPO laser system (laser pulse duration, 7 ns; pulse energy, 20 mJ; repetition rate, 0.2 Hz) into an optical fiber inserted at its other end into the NMR tube via a coaxial insert. Photo-CIDNP difference spectra were recorded directly by using a pre-saturation pulse train to destroy thermal polarization prior to the laser pulse.[49]

Upon photoexcitation, hyperpolarized ^{1}H resonances are observed from both 5-deazaFMN and tryptophan. Positive and negative signals represent emissive and enhanced absorptive transitions, respectively. Signal intensities and linewidths are comparable to those reported from photoreaction of FMN with tryptophan under comparable experimental conditions.[44] Nuclear hyperpolarization of the protons in the redox-active aromatic moiety of 5-deazaFMN is relevant for the identification of radicals: H8α and H9 exhibit absorptive polarization; resonances in emission are observed for H5, H6 and H7α, see Figure 2. Clearly, observation of hyperpolarization in solutions of 5-deazaFMN and tryptophan provides unambiguous evidence of the occurrence of radical species of these constituents formed in the course of photochemical processes.

The signs of the polarized NMR resonances can be rationalized using Kaptein's rule for the net polarization

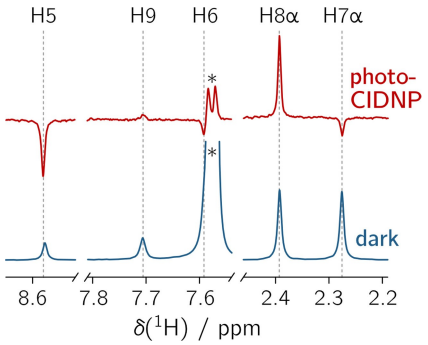

Figure 2. Thermally polarized (blue) and photo-CIDNP (red) ^{1}H NMR spectrum of 5-deazaFMN at pH 6.6. Only ^{1}H resonances of the aromatic moiety are shown except for the resonance of H4 of L-tryptophan (labelled with an asterisk). For the photo-CIDNP experiment the sample was irradiated with laser pulses of 420 nm. All resonances were referenced to the HDO signal at 4.7 ppm.

Γ_i.[50] Γ_i results from the product of four signs and yields either "+" or "−" for an absorptive or an emissive signal, respectively:

$$\Gamma_i = \mu \times \varepsilon \times \mathrm{sgn}(\Delta g) \times \mathrm{sgn}(A_{\mathrm{iso},i}). \quad (1)$$

For a triplet precursor of radical pair formation, the parameter μ is "+"; for a singlet precursor it is "−". Depending on the observed product, recombination/reencounter or escape, the parameter ε is "+" or "−", respectively. The sign of the difference in isotropic g values of the two radicals, $\Delta g = g_{\mathrm{iso},1} - g_{\mathrm{iso},2}$, is also relevant, as are the signs of isotropic hyperfine coupling constants $A_{\mathrm{iso},i}$ describing the interaction of proton spin i with the unpaired electron spin of the respective radical of the radical pair.

In order to verify that Kaptein's rule applies to the investigated radical species of 5-deazaFMN and tryptophan forming the radical pair, all parameters were determined as follows. (i) As our experimental set-up limits the time resolution to 2.5 μs, the measured CIDNP polarization can be attributed to recombination products only. In this case, the parameter ε is positive. (ii) Net polarizations Γ_i of the 5-deazaFMN's aromatic protons H8α and H9 are positive, whereas the protons H5, H6 and H7α reveal negative net polarizations. (iii) Due to a lack of experimental data, values for A_{iso} and g_{iso} of relevant 5-deazaFMN radicals (anionic 5-deazaFMN$^{\bullet-}$ and the neutral 5-deazaFMN radical protonated at N1, subsequently denoted 5-deazaFMN(H1)$^{\bullet}$) were predicted using density functional theory (DFT). For all protons i attached to the aromatic ring, $A_{\mathrm{iso},i}$ was found to be of the same sign as the polarization Γ_i of the respective CIDNP resonance, i.e., $\Gamma_i = +\mathrm{sgn}(A_{\mathrm{iso},i})$. According to Eqn. (1) this requires $\mu \times \mathrm{sgn}(\Delta g) =$ "+". (iv) For 5-deazaFMN-(H1)$^{\bullet}$ and 5-deazaFMN$^{\bullet-}$, g_{iso} values of 2.002752 and 2.002799 were calculated using a polarizable continuum model to mimic unspecific water solvation, respectively. Further refinement by positioning H_2O (or D_2O) molecules specifically around the aromatic moiety of the 5-deazaFMN structure did not alter these results significantly. Figure S3 shows the optimized structures used for the DFT calculations. All g_{iso} and A_{iso} values of the relevant ^{1}H nuclei are given in Table S1. (v) For the neutral tryptophanyl radical (Trp$^{\bullet}$) a g_{iso} of 2.0026 was measured,[51] whereas—to the best of our knowledge—no such experimental data exist for the tryptophanyl cation radical (TrpH$^{\bullet+}$) expected to be formed at pH 6.6. Works based on DFT calculations predict a value of 2.00280 for TrpH$^{\bullet+}$,[52] which is almost identical to the DFT-calculated g_{iso} values of both 5-deazaFMN radical species, 5-deazaFMN(H1)$^{\bullet}$ and 5-deazaFMN$^{\bullet-}$. Hence, the sign of the difference of the g_{iso} values of the 5-deazaFMN radical and TrpH$^{\bullet+}$ cannot be determined with certainty, as subtle variations of the simulated geometries may yield "+" or "−" for this parameter with implications for the sign of μ, thus leading to an ambiguity about the precursor multiplicity of radical pair generation. (vi) To resolve this issue, we performed additional hyperpolarization experiments with a mixture of 5-deazaFMN and tyrosine (Tyr) instead of tryptophan, see Figure S4. Regardless of their protonation state, the g_{iso} values of tyrosine radicals are generally larger

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(≈2.0045) than those of both 5-deazaFMN radicals considered,[53–54] thus yielding sgn(Δg) = "−". As compared to the photo-CIDNP polarizations shown in Figure 2, all ^{1}H resonances of 5-deazaFMN are reversed in the photoreaction with tyrosine, hence: $\Gamma_i = -\mathrm{sgn}(A_{\mathrm{iso},i})$. This requires $\mu \times \mathrm{sgn}(\Delta g) =$ "−", and consequently $\mu =$ "+". Hence, the detected 5-deazaFMN radical is produced by photoreduction of the triplet-state of 5-deazaFMN. (vii) Given that $\mu =$ "+" requires sgn(Δg) = "+" to yield the obtained relation $\Gamma_i = +\mathrm{sgn}(A_{\mathrm{iso},i})$ for the photoreaction of 5-deazaFMN with tryptophan. Therefore, we conclude that the isotropic g_{iso} values of both 5-deazaFMN radicals must be larger than that of Trp$^\bullet$ and also of TrpH$^{\bullet+}$, which means that either the DFT-calculated g_{iso} value of TrpH$^{\bullet+}$ (2.00280)[52] is too large or the respective values of 5-deazaFMN(H1)$^\bullet$ (2.002752) and 5-deazaFMN$^{\bullet-}$ (2.002799) predicted are slightly too small.

In order to extract experimental relative A_{iso} values and to further characterize the protonation state of the generated 5-deazaFMN radical, the calculated A_{iso} values were correlated with relative CIDNP intensities.[39] At pH 6.6, see Figure 3, an exceptionally good correlation is found for both radical species: 5-deazaFMN(H1)$^\bullet$ ($R^2 = 0.9899$) and 5-deazaFMN$^{\bullet-}$ ($R^2 = 0.9937$). This is unambiguous evidence of the fact that a one-electron reduced 5-deazaFMN species is generated in the course of the photo-initiated redox reaction with tryptophan. As both correlations are close to 1, the distinction between protonated (5-deazaFMN(H1)$^\bullet$) and anionic (5-deazaFMN$^{\bullet-}$) radical based on the measured ^{1}H intensities proves to be difficult. The pK_{A} of the 5-deazalumiflavin radical was determined to 6,[4] which would suggest an anionic radical at the pH of 6.6 although this is not readily applicable to 5-deazaFMN.

The hyperfine coupling constants of the 5-deazaFMN radical found can be compared to values of neutral and anionic FMN radicals already published.[44,55] The hyperfine structure of the FMN radicals differs significantly due to protonation of N5 and can thus be differentiated unambiguously by photo-CIDNP measurements. Comparison of ^{1}H hyperfine coupling constants shows that the 5-deazaFMN radical species resembles the anionic FMN radical whereas its hyperfine structure is hardly influenced by protonation of N1. Comparison of fit results of CIDNP experiments at different pH values, see Table S2 and Figure S6, shows that at pH 1.5, the protonated radical 5-deazaFMN(H1)$^\bullet$ yields the highest correlation. This coincides with the known pK_{A} of the lumiflavin analogue and its, above mentioned, triplet state. At all higher pH values, however, the A_{iso} of the 5-deazaFMN$^{\bullet-}$ radical correlate best with the measured ^{1}H CIDNP intensities.

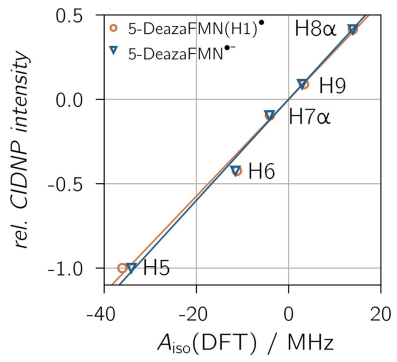

Figure 3. Correlation plot of relative ^{1}H photo-CIDNP intensities of 5-deazaFMN and calculated isotropic hyperfine coupling constants of the protonated and anionic 5-deazaFMN radical species (see Figure S3 for structures). CIDNP intensities were extracted from the experiment in Figure 2 by fitting Lorentzian and Gaussian functions to the CIDNP signals. The linear regression fits were forced to go through the origin. The fits resulted in slopes of 0.0287 MHz^{-1} and 0.0299 MHz^{-1} and R^2 values of 0.9899 and 0.9937 for 5-deazaFMN(H1)$^\bullet$ and 5-deazaFMN$^{\bullet-}$, respectively.

The similarity of both radical species is illustrated by their singly occupied molecular orbitals (SOMOs), see Figure 4. Negative and positive signs of the wavefunction are colored in blue and red, respectively. A small SOMO amplitude is observed for both H7α and H9 which yield small hyperfine couplings in 5-deazaFMN(H1)$^\bullet$ and 5-deazaFMN$^{\bullet-}$. Compared to this a higher amplitude is exhibited by H6 and H8α. The highest amplitude by far is on H5 which correlates with the strong hyperfine coupling for this proton: DFT calculations predict A_{iso} values of −34.13 MHz and −35.99 MHz for 5-deazaFMN$^{\bullet-}$ and 5-deazaFMN(H1)$^\bullet$, respectively. For all other nuclei, the calculated A_{iso} are comparable in size for 5-deazaFMN(H1)$^\bullet$ and 5-deazaFMN$^{\bullet-}$ which complicates the differentiation of the protonation state of the radical based on ^{1}H photo-CIDNP. However, the radicals clearly differ in the SOMO amplitude on N1 and N3. While the anionic radical exhibits a significant SOMO amplitude on both atoms, the protonated radical lacks SOMO amplitude on these positions. Based on these calculations, photo-CIDNP experiments with ^{15}N as well as ^{13}C enriched 5-deazaflavins may be helpful to further characterize its protonation state.

Comparing the Mulliken spin populations, 5-deazaFMN$^{\bullet-}$ and 5-deazaFMN(H1)$^\bullet$ only show small deviations for ^{1}H nuclei (see Table S5 and Figure S7). A comparison with the neutral and anionic FMN radicals, FMN(H5)$^\bullet$ and FMN$^{\bullet-}$ respectively, again shows a similar spin density distribution of both 5-deazaFMN radicals with the anionic FMN radical species.

A direct comparison of the electronic structures of the 5-deazaFMN radical presented in this study and the radical of the C5-phenylated 5-deazaflavin derivative[21] by means of

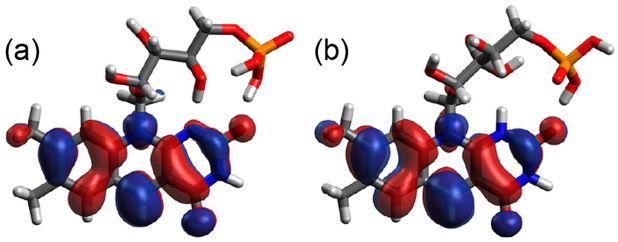

Figure 4. SOMO of the 5-deazaFMN$^{\bullet-}$ radical (a) and the 5-deazaFMN(H1)$^\bullet$ radical (b). Negative and positive amplitudes of the frontier orbitals are colored blue and red, respectively.

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hyperfine data is hampered by the fact that proton couplings of H6 and H9 present in both structures are not resolved in the cw EPR spectrum recorded from the latter. The two reported couplings from the latter radical were assigned to two protons in the phenyl ring attached to C5 of the 5-deazaflavin derivative. These are comparable in size with those of H6 and H9 in the 5-deazaFMN radical reported here (see Figure 3), thus indicating that substantial unpaired electron spin density is shifted into the phenyl substituent in the derivative. Whether such delocalization is a prerequisite for the catalytic efficiency of 5-deazaflavins in photocatalysis or not clearly requires further investigation, for which the application of photo-CIDNP NMR may prove particularly valuable.

Conclusion

Since the first report of the catalytic role of F_o,[56] 5-deazaflavins have been described as obligate two-electron transfer agents. Their redox reactivity has been seen as analogous to nicotinamides rather than flavins.[12,57] Now, with the presented unambiguous evidence of a 5-deazaFMN radical via photo-CIDNP experiments presented here, the reactivity of 5-deazaFMN is extended to one electron transfer reactions. This ultimately sheds new light on the catalytic role of 5-deazaflavins in natural redox processes.

Supporting Information

The authors have cited additional references within the Supporting Information.[58–67]

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: 5-Deazaflavin · Dynamic Nuclear Polarization · Hyperfine Coupling · Photocatalysis · Spin-Correlated Radical Pairs

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