BIOORGANIC CHEMISTRY 6, 157–163 (1977)

A New Spin-Labelled Analogue of Nicotinamide Adenine Dinucleotide

MOHAMED A. ABDALLAH,* JEAN-JACQUES ANDRE,† AND JEAN-FRANÇOIS BIELLMANN*

Laboratoire de Chimie Organique Biologique, associé au CNRS, Institut de Chimie, Université Louis Pasteur, 1 rue Blaise Pascal, 67008 Strasbourg, France, and †Centre de Recherches sur les Macromolécules, CNRS, 6 rue Boussingault, 67083 Strasbourg, France

Received October 5, 1976

The preparation of a spin-labelled analogue of nicotinamide adenine dinucleotide, 3-(4′,4′,5′,5′-tetramethyl-3′-oxide-1′-oxyl-2′-imidazolinyl) pyridine adenine dinucleotide, is described. This compound was obtained by treatment of 3-carboxaldehyde pyridine adenine dinucleotide with 2,3-dimethyl-2,3-dihydroxylaminobutane followed by oxidation with lead dioxide. The interpretation of the particular electron spin resonance spectra of this nitronylnitroxide (five lines) in terms of the rotational correlation time of the radical is shown to be possible. The high stability of this compound makes its use in NAD^+-dependent biological systems feasible.

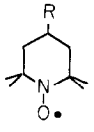
INTRODUCTION

Spin-labels have been proven to be very useful tools for the study of biological systems (*1*). However, this utility relies on the availability and stability of spin-labelled compounds, which in some cases may be difficult to prepare. A few years ago, a stable spin-labelled analogue **1** of ADPR[1] was prepared and used in the study of some dehydrogenases (*2–4*). More recently, analogues **2a** of 3′:5′-cyclic AMP (*5*) and **2b** of NAD^+ (*6*) having a spin-label on the adenine ring were used as probes in the study of cAMP-dependent protein kinases and dehydrogenases, respectively, whereas spin-labelled analogue **3** of acetyl CoA appeared to be very useful in the study of pig heart citrate synthase (*7*).

In order to obtain more information on the nicotinamide ring environment in NAD^+-dependent biological systems, we synthesized and studied some physical properties of a stable spin-labelled NAD^+ analogue **4** possessing a nitronylnitroxide group at C-3 of the pyridinium ring.

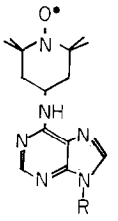
[1] *Abbreviations* used: to^{3}PdAD$^+$, 3-(4′,4′,5′,5′-tetramethyl-3′-oxide-1′-oxyl-2′-imidazolinyl)pyridine adenine dinucleotide; 5′-AMP, adenosine 5′-monophosphate; cAMP, adenosine 3′:5′-cyclic monophosphate; ADPR, adenosine diphosphoribose; al^{3}PdAD$^+$, 3-carboxaldehyde pyridine adenine dinucleotide; s-NAD^+, thionicotinamide adenine dinucleotide; cn^3-PdAD$^+$, 3-cyano pyridine adenine dinucleotide; esr, electron spin resonance; uv, ultraviolet spectrophotometry.

ISSN 0045-2068



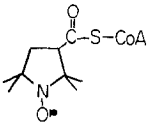
1

R = adenosinediphosphoribosyl

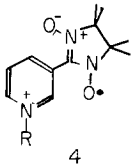


2

2a, R = 3′,5′-cyclic ribosyl phosphate
2b, R = nicotinamideribosediphosphoribosyl



3



4

to ^{3}PdAD$^+$
R = adenosinediphosphoribosyl

FIGURE 1

RESULTS AND DISCUSSION

The methods available for the preparation of NAD$^+$ analogues substituted on the nicotinamide are:

(1) Synthesis of the nucleoside by reaction of a heterocyclic base with an activated ribose, followed by phosphorylation and coupling with 5′-AMP.

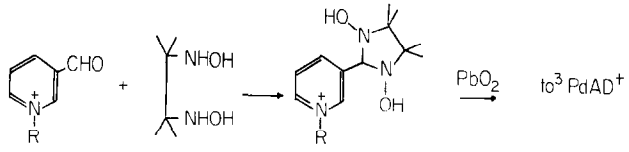
(2) Enzymic transglycosidation with NAD$^+$ glycohydrolase from a mammalian source (*8–14*).

(3) Chemical reaction on NAD$^+$ or on NAD$^+$ analogues.

An example of the first method is the total synthesis of NAD$^+$ (*15*, *16*). The last method has been rather seldom used, but includes preparation of tetrahydro-NAD by catalytic reduction of NADH (*17*), transformation of s-NAD$^+$ to 3-cyanopyridine AD$^+$ (*18*), the Hofmann degradation of NAD$^+$ to 3-aminopyridine AD$^+$ (*19*) and further transformation to 3-diazopyridine AD$^+$ (*9*, *14*) and 3-halogenopyridine AD$^+$ (*14*).

In order to prepare the spin-labelled analogue **4**, we tried (unsuccessfully) the enzymic transglycosidation of NAD$^+$ with 3-(4′,4′,5′,5′-tetramethyl-3′-oxide-1′-oxyl-2′-imidazolinyl) pyridine **8** (*20*). Therefore, we applied to an analogue of NAD$^+$, al^{3}PdAD$^+$, the synthesis of nitronylnitroxides starting from aldehydes, as described by Ullmann and co-workers (*21*), but with addition of some modifications due to the use of water as a solvent.

al^{3}PdAD$^+$ was prepared by treatment of NAD$^+$ with 3-carboxyaldehyde pyridine in the presence of pig brain NAD$^+$ glycohydrolase (*11*). A purification on Dowex 1 × 2 (formate) gave a large fraction of this analogue contaminated with some residual NAD$^+$. The impure 3-carboxaldehyde pyridine AD$^+$ was treated with an excess of 2,3-dimethyl-2,3-dihydroxylaminobutane (*22*) until no al^{3}PdAD$^+$ was detected. After three chromatographies on Dowex 1 × 2, analogue **6** was obtained free of NAD$^+$ in 26% yield (with respect to the total amount of NAD$^+$). Oxidation with lead dioxide of analogue **6** in aqueous solution yielded the spin-label **4** after purification, (50–60%).

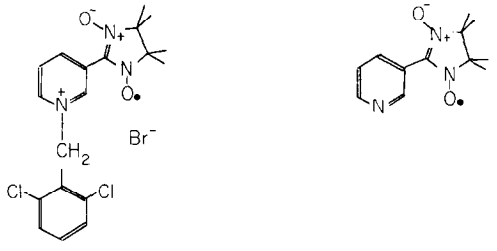


5 6 4

R = adenosinediphosphoribosyl

FIGURE 2

For comparison, we also prepared 1-(2′,6′-dichlorobenzyl)-3-(4′,4′,5′,5′-tetramethyl-3′-oxide-1′-oxyl-2′-imidazolinyl)pyridinium bromide **7** by treatment of pyridine **8** (*20*) with 2,6-dichlorobenzyl bromide.



7 8

FIGURE 3

Optical Spectra

The absorption spectra of the three compounds **4**, **7**, and **8** show two characteristic bands at around 580 and 365 nm. However, the complex patterns observed in hexane for related nitronylnitroxides (*21*) are very poorly resolved in methanolic or aqueous solution (broad shoulders). The absorption at higher wavelength (580–610 nm) has been attributed to an n–π^* transition with a characteristically low molar extinction coefficient (100–200 $M^{-1}\cdot$liter$\cdot$cm^{-1}), whereas the absorption at 365 nm is presumably a π–π^* transition with a high ε value ($\simeq$ 10 000).

Moreover, the presence of the positive charge on the pyridinium ring in compounds **4** and **7** causes a decrease of the wavelength of these two bands compared to the corresponding bands in **8** (Table 1).

Electron Spin Resonance Spectra

The esr spectra of compounds **4**, **7**, and **8** are characteristic of nitronylnitroxide spectra as described by Ullmann *et al.* (*21*) (Table 1). The five lines correspond to two equivalent nitrogen atoms and have their intensities in the ratio 1:2:3:2:1.

The mean g factors of the three nitroxides **4**, **7**, and **8** are very similar (2.0065) but slightly different from that reported for some other nitronylnitroxides. The linewidth is due to the unresolved hyperfine splitting, not only from the 12 hydrogen atoms of the methyl groups (*21*) but also from the aromatic substituent (*20*). The large hyperfine

TABLE 1

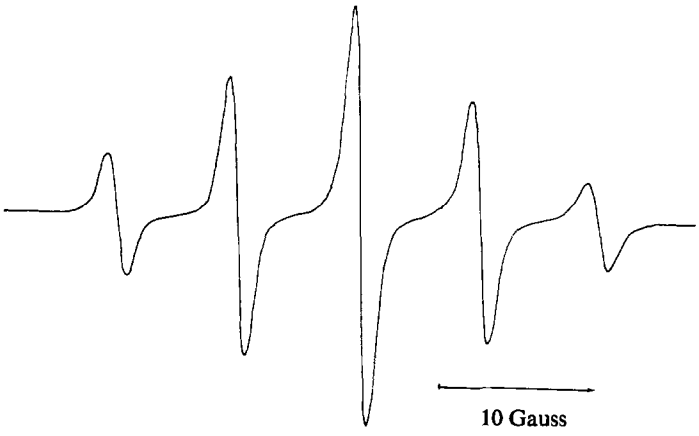
ELECTRON SPIN RESONANCE AND OPTICAL DATA OF THE RADICALS **4**, **7**, AND **8**[a]

Compound	g factor	a_N (gauss)	ΔH (gauss)	λ_{max} (nm)[b]	
				H_2O (pH 7.5)	Methanol
4	2.0065	7.85	1.38	360 (8800), 580 (220)	—
7	2.0065	7.85	1.37	359 (9200), 580 (220)	366 (1100), 610 (120)
8	2.0066	8.04	1.20	355 (9800), 557 (810), 348 (shoulder)	364 (1100), 585 (470), 352 (shoulder)

[a] g factor, hyperfine splitting constant, linewidth of the central line, wavelength of the maximum of the two characteristic absorption bands.

[b] The molar extinction coefficient ε is expressed as $mole^{-1} \cdot liter \cdot cm^{-1}$ and is given in parentheses.

constant a_N determined for both nitrogen atoms is slightly decreased on replacing the pyridine in **8** by a pyridinium in **4** and **7** independently of the nature of substituent on the N-1 of the pyridinium ring.



e.s.r spectrum of to $^3PdAD^+$, 1.2 10^{-4}M in 0.1M phosphate buffer pH 7.5

FIGURE 4

Nitronylnitroxides as Potential Spin Labels

Although more complex than the simple nitroxide radicals **1–3**, nitronylnitroxides may be used as spin labels. As for nitroxide radicals, the rapid tumbling of the molecule deeply perturbs the esr spectra. The variation of the different linewidth may be related to the rotational correlation time τ_c of the molecule.

A relation similar to that used for the interpretation of simple nitroxide spectra (*1*, *23*, *24*) may be found for nitronylnitroxides. The only important modification arises from the evaluation of the average value of the total nuclear spin of the system $\langle J(J+1) \rangle M$ for a given value of M, the nuclear spin quantum number. This number is $I(I+1)$ for one nucleus of spin I, but is 6, 4, 8/3, respectively, when $|M| = 2, 1, 0$ for two nuclei of spin 1 (*23*).

From Stone and co-workers (*24*), in the case of Lorentzian shape lines, the linewidth parameter T_2^{-1} may be expressed as

$$T_2^{-1}(M) = \tau_c\{[3\langle J(J+1)\rangle M + 5M^2](b^2/40) + \beta B^2 + \alpha BM\} + X \qquad (1)$$

with $M = -2$ to $+2$ (Eqs. (1a) to (1e)). In these equations:

(i) B is the applied field strength, in gauss.

(ii) β is a term depending on the anisotropy of the g factor and it is found to be negligible.

(iii) The term α depends simultaneously on the anisotropy of the g factor and on the anisotropy $A_{zz} - A_{xx}$ of the hyperfine constants A. This term is not negligible but can be evaluated from the esr linewidth increase with increasing field.

(iv) $b = 4\pi/3(A_{zz} - A_{xx})$ (*24*).

(v) X represents all the other contributions to the relaxation (independently on M).

From the five relations (1a)–(1e), after elimination of the X contribution, one can write four expressions and then calculate the correlation time τ_c from the absolute value of the central line's width T_2^{-1} (0) and the square roots of the ratios of the experimental derivative curve peak heights $T_2(0)/T_2(M)$.

Such a tumbling effect is very small on the small molecules such as **7** or **8**, but is already important for to^{3}PdAD$^+$ in aqueous solution at room temperature (viscosity, $\eta = 1$ cP) as shown in Fig. 4.

Though we observe a slight deviation from the Lorentzian shape in the spectra (due to unresolved hyperfine structures), the foregoing description of the tumbling was used as an evaluation of the correlation time of the spin label **4**. Furthermore, since no data are available on the experimental principal values of the hyperfine interaction and g factor tensors, we used a value of 13 G for the anisotropy of the hyperfine constant (about half of the value found for nitroxides of type **1**–**3**).

Thus from Eq. (1a)–(1e), we could calculate a correlation time of 2×10^{-10} sec, which is a reasonable value for such a molecule.

The use of nitronylnitroxide as a spin label is related to the possibility of interpreting the modifications of the spectra, as is commonly done for the simple nitroxides (*1–3*). The esr study shows that such an interpretation is possible for nitronylnitroxides spin labels. Therefore, and due to the high stability of to^{3}PdAD$^+$, its use as a spin-label probe in biological systems can be envisioned.

EXPERIMENTAL

NAD$^+$ glycohydrolase from pig brain originated from Sigma Chemical Company (St. Louis, MO.).

Ultraviolet spectra and kinetics were measured on a Varian Cary 118 spectrophotometer.

The esr measurements were performed on a Varian V 4502 spectrometer using a 104 mode resonating cavity. The spectra were obtained at 20°C with an aqueous solution cell for variable temperature accessories (Varian type E248-1). Quantitative data were measured with respect to "pitch" as an external standard, whereas g factors and hyper-

fine splitting constants a_N were determined with potassium peroxylaminodisulfonate as an external standard.

Preparation of 1-(2',6'-Dichlorobenzyl)3-(4',4',5',5'-tetramethyl-3'-oxide-1'-oxyl-2'-imidazolinyl)Pyridinium Bromide

2,6-Dichlorobenzyl bromide (0.25 g; 1.04 m*M*) was added to a solution of 3-(4',4',5',5'-tetramethyl-3'-oxide-1'-oxyl-2'-imidazolinyl)pyridine (0.245 g; 1.05 mM) (*22*) in acetone (2ml). After 72 hr of stirring at room temperature, the green crystals were filtered, washed with ether, and recrystallized in ethanol–ether (1:2; 408 mg; 83%).

λ_{max} (methanol) = 283, 278.5, 243 nm (ε = 10 350), 366 nm (ε = 11 100), 610 nm (ε = 125).

λ_{max} (H_2O, pH 7.5) = 237 (ε = 4800), 273 (ε = 9200), 280 (shoulder, ε = 8400), 359 (ε = 9200), 580 (ε = 220).

Found: C%, 48.19; H%, 4.57; N%, 9.00. $C_{19}H_{21}N_3O_2Cl_2Br$ requires C%, 48.12; H%, 4.46; N%, 8.86. For esr, see Table 1.

Preparation of 3-(4',4',5',5'-Tetramethyl-1',3'-dihydroxy-2'-imidazolinyl)Pyridine AD⁺ **6**

Freshly distilled 3-carboxaldehyde pyridine (8 g) was added to NAD^+ (8 g) dissolved in 0.1 M phosphate buffer, pH 7.3 (160) ml. The pH was adjusted to 7.3 by addition of 1 *M* dipotassium hydrogen orthophosphate (ca. 60 ml), and NAD^+ glycohydrolase from pig brain (2.2 g) was added. The suspension was kept at 37°C and stirred under argon for 18 hr.

After acidification to pH 4 by addition of solid trichloroacetic acid, the suspension was centrifuged, and the supernatant was diluted to 400 ml, chromatographed on a Dowex 1 × 2 formate column (3.8 × 52), and eluted with a linear gradient of water–0.4 *N* formic acid (8 liters, total volume). The fractions containing al^3PdAD^+ (*11*) contaminated with some NAD^+ were pooled and lyophylized (5 g).

A solution of 1 *M* dipotassium hydrogen orthophosphate (ca. 10 ml) was added to the foregoing mixture dissolved in water (25 ml) in order to adjust the pH to 6.0. 2,3-Dimethyl-2,3-dihydroxylaminobutane monosulfate (*22*) (5 g) was then added, and the solution was stirred for 3.5 hr, until no al^3PdAD^+ was enzymatically detected. The solution was then chromatographed on a Dowex 1 × 2 formate column (2.8 × 72). The column was first eluted with 0.5 liters of water and then with a linear gradient of water–0.4 *N* formic acid (4 liters, total amount). After three chromatographies in identical conditions, the yield of 3-(4',4',5',5'-tetramethyl-1',3'-dihydroxy-2'-imidazolinyl)-$PdAD^+$ was 2.5 g (26%). The compound was free of NAD^+

λ_{max} = 257 nm ε = 18 600
pH 7.5

Found: C%, 38.06; H%, 5.61; N%, 13.24. $C_{27}H_{40}N_8O_{15}P_2$, 4 H_2O requires C%, 38.12; H%, 5.69; N%, 13.17.

Preparation of the Spin-labelled Analogue **4**

Lead dioxide (0.65 g) was added to a solution of 3-(4',4',5',5'-tetramethyl-1',3'-dihydroxy-2'-imidazolinyl)$PdAD^+$ **6** (0.5 g) dissolved in water (30 ml) and the suspension was vigorously stirred in the dark for 24 hr. After addition of formic acid (0.4 *N*,

7 ml), the suspension was filtered and the solution was chromatographed on a Dowex 1 × 2 formate column (2 × 40) eluted with a linear gradient of water–0.4 *N* formic acid (2 liters, total amount) to yield, after lyophylization, 0.27 g (54%) of to^{3}PdAD$^+$.

uv pH 7.5 λ_{max} 257 $\varepsilon = 21\,000$
λ_{max} 360 $\varepsilon = 8800$
λ_{max} 580 $\varepsilon = 210$

Found: C%, 38.13; H%, 5.34; N%, 13.18. $C_{27}H_{37}N_8O_{15}P_2$, 4 H_2O requires C%, 38.25; H%, 5.35; N%, 13.22. For esr, see Table 1.

ACKNOWLEDGMENTS

This work was supported in part by the DGRST (Contract No. 73-7-1312) and the Fondation pour la Recherche Médicale Française.

REFERENCES

1. H. M. SWARTZ, J. R. BOLTON, AND D. C. BORG, "Biological Applications of Electron Spin Resonance," Wiley, New York, 1972.
2. H. WEINER, *Biochemistry* **8**, 526 (1969).
3. (*a*) A. S. MILDVAN AND H. WEINER, *Biochemistry* **8**, 552 (1969); (b) A. S. MILDVAN AND H. WEINER, *J. Biol. Chem.* **244**, 2465 (1969).
4. W. BALTHASAR, *Eur. J. Biochem.* **22**, 158 (1971).
5. J. HOPPE AND K. G. WAGNER, *Eur. J. Biochem.* **48**, 519 (1974).
6. W. E. TROMMER, H. WENZEL, AND G. PFLEIDERER, *Ann.* 1357 (1974).
7. S. W. WEIDMAN, G. R. DRYSDALE, AND A. S. MILDVAN, *Biochemistry* **12**, 1874 (1973).
8. B. M. ANDERSON, J. C. CIOTTI, AND N. O. KAPLAN, *J. Biol. Chem.* **234**, 1219 (1959).
9. B. M. ANDERSON AND N. O. KAPLAN, *J. Biol. Chem.* **234**, 1226 (1959).
10. A. M. STEIN, J. K. LEE, C. D. ANDERSON, AND B. M. ANDERSON, *Biochemistry* **2**, 1015 (1963).
11. N. O. KAPLAN, M. M. CIOTTI, AND F. E. STOLZENBACH, *J. Biol. Chem.* **221**, 833 (1956).
12. N. O. KAPLAN AND M. M. CIOTTI, *J. Biol. Chem.* **221**, 823 (1956).
13. J. F. BIELLMANN, G. BRANLANT, B. Y. FOUCAUD, AND M. J. JUNG, *FEBS Lett.* **40**, 29 (1974).
14. M. A. ABDALLAH, J. F. BIELLMANN, J. P. SAMAMA, AND A. D. WRIXON, *Eur. J. Biochem.* **64**, 351 (1976).
15. N. A. HUGHES, G. W. KENNER, AND A. TODD, *J. Chem. Soc.* 3733 (1957).
16. CH. WOENCKHAUS, *Fortsch. Chem. Forsch.* **52**, 209 (1974).
17. J. F. BIELLMANN AND M. J. JUNG, *Eur. J. Biochem.* **19**, 130 (1971).
18. J. F. BIELLMANN AND M. J. JUNG, *FEBS Lett.* 7, 199 (1970).
19. T. L. FISCHER, S. V. VERCELLOTTI, AND B. M. ANDERSON, *J. Biol. Chem.* **248**, 4293 (1973).
20. M. S. DAVIS, K. MOROKUMA, AND R. W. KREILICK, *J. Amer. Chem. Soc.* **94**, 5588 (1972).
21. E. F. ULLMANN, J. H. OSIECKI, D. G. B. BOOCOCK, AND R. DARCY, *J. Amer. Chem. Soc.* **94**, 7049 (1972).
22. M. LAMCHEN AND T. W. MITTAG, *J. Chem. Soc. C* 2300 (1966).
23. D. KIVELSON, *J. Chem. Phys.* **33**, 1094 (1960).
24. T. J. STONE, T. BUCKMAN, P. L. NORDIO, AND H. M. MCCONNELL, *Proc. Nat. Acad. Sci. USA* **54**, 1010 (1965).