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Determination of the transglycosidation activity of NAD^+ glycohydrolases with 4-(2′-alkyl-sulfanyl-vinyl)-pyridine derivatives generating chromophoric NAD^+ analogs

Karine Pacaud, Denis Tritsch,* Alain Burger, and Jean-François Biellmann

Laboratoire de Chimie Organique Biologique, UMR7123, Faculté de Chimie, Université Louis Pasteur, 1 rue Blaise Pascal, FR-67008 Strasbourg, Cedex, France

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Abstract

The base exchange of nicotinamide with pyridine derivatives **1a**–**5a**, catalyzed by pig brain NAD^+ glycohydrolase and ADP-ribosyl cyclase from *Aplysia californica*, generated the corresponding NAD^+ analogs **1b**–**5b**. These analogs exhibited a high absorbance band in the visible region. The transglycosidation rate was determined by monitoring the absorbance increase. Among the tested derivatives, (*E*)-4-[2-(methylsulfanyl)-vinyl]-pyridine **1a** was the most suitable substrate for pig brain NAD^+ glycohydrolase while 4-[1,3]-dithiolan-2-ylidenemethyl-pyridine **3a** was the most efficient for ADP–ribosyl cyclase from *A. californica*.

Keywords: 4-(2′-Alkyl-sulfanyl-vinyl)-pyridine; NAD^+ analogs; NAD^+ glycohydrolase; ADP-ribosyl cyclase; NADase; Transglycosidation; Push–pull double bond

* Corresponding author. Fax: +33-3-90-24-13-45.
E-mail address: dtritsch@chimie.u-strasbg.fr (D. Tritsch).

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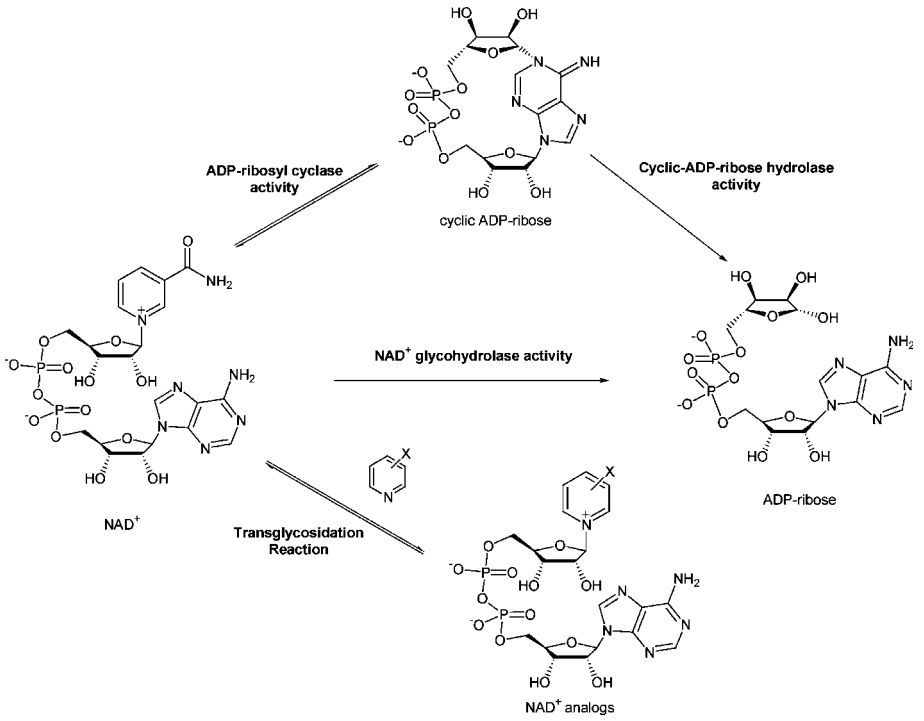


Scheme 1. Multicatalytic activity of NAD^+ glycohydrolases.

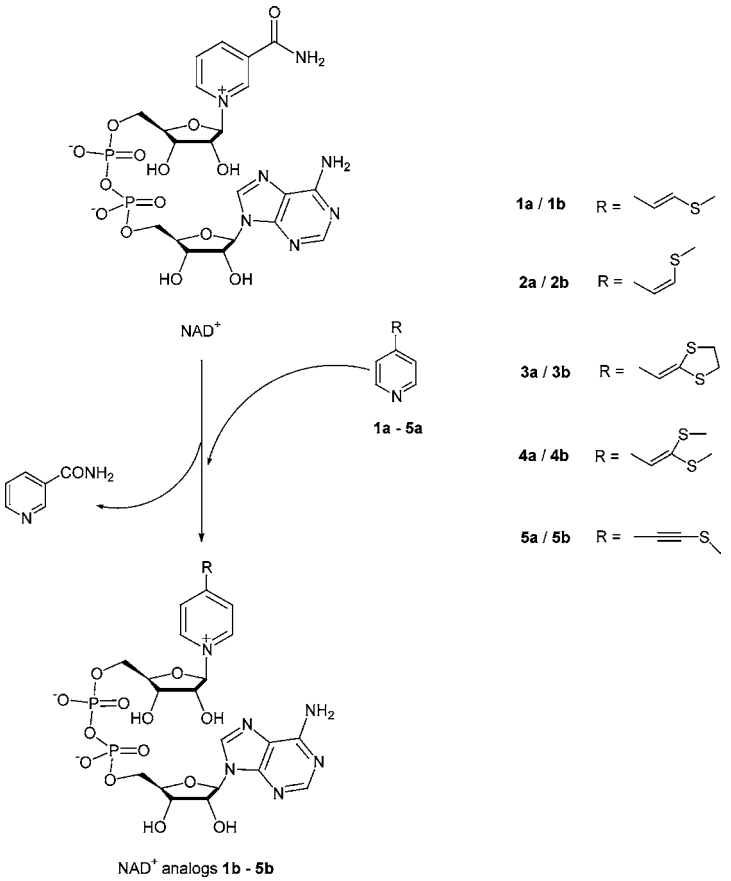
1. Introduction

$NAD(P)^+$ glycohydrolases (NADases;[1] EC 3.2.2.5 and EC 3.2.2.6) constitute a heterogeneous family of enzymes. They catalyze the hydrolytic cleavage of the *N*-glycosidic bond of β-NAD^+ leading to the formation of an ADP-ribosyl oxocarbenium ion. Then this intermediate could evolve according to three competing pathways (Scheme 1). The reaction between ribosyl C-1′ and water gives ADP–ribose. Intramolecular cyclization between ribosyl C-1′ and N-1 of the adenine ring produces cyclic ADP–ribose (cADPR). In the presence of pyridine derivatives, NAD^+ analogs are obtained via a transglycosidation reaction [1,2]. In fact NADases have multicatalytic activities and the yield in each product depends on the enzyme and the reaction conditions. Mammalian "classical" NADases transform NAD^+ essentially into ADP–ribose. Only small proportions of cADP–ribose (less than 2% of the reaction products) are detected [3,4]. On the other hand ADP–ribosyl cyclase from *Aplysia californica* produces almost exclusively cADPR [5,6]. Moreover in the presence of nicotinic acid, this enzyme catalyzes the exchange of the nicotinamide group of

[1] *Abbreviations used*: NADase(s), $NAD(P)^+$ glycohydrolase(s); c-ADPR, cyclic adenosine diphosphate ribose; $NAAD(P)^+$, nicotinic acid adenine dinucleotide (phosphate).

$NAD(P)^+$ with nicotinic acid to give nicotinic acid adenine dinucleotide (phosphate) [7]. The interest in NADases is growing since the discovery that cADPR and nicotinic acid adenine dinucleotide phosphate ($NAADP^+$) have intracellular Ca^{2+}-mobilizing properties (for a review, see [8]).

4-(2′-Alkyl-sulfanyl-vinyl)-pyridinium salts absorb strongly in the near visible region whereas the corresponding (2′-alkyl-sulfanyl-vinyl)-pyridines do not [9]. These spectral properties were at the origin of the development of new specific cysteine reagents [9]. As the enzyme exchanges nicotinamide with a large variety of pyridine derivatives [10,12] we developed a continuous spectrophotometric assay allowing the determination of the transglycosidation rate of NADases. It is based on the base exchange between nicotinamide and (2′-alkyl-sulfanyl-vinyl)-pyridines generating NAD^+ analogs absorbing, as the corresponding 4-(2′-alkyl-sulfanyl-vinyl)-pyridinium salts, in the visible region. The enzymatic activity could be determined by following the absorbance increase due to the formation of these NAD^+ analogs.



Scheme 2. Transglycosidation reaction of NADase with pyridine derivatives **1a**–**5a**.

Pyridine derivatives **1a**–**5a** were synthesized and tested on pig brain NADase and ADP-ribosyl cyclase from *A. californica*. The kinetic constants of the transglycosidation reaction were determined and the generated NAD^+ analogs **1b**–**5b** characterized (Scheme 2).

2. Materials and methods

Thin-layer chromatography was performed on silica analytical Si 60 F_{254} plates (Merck), flash chromatography on silica gel Si 60 230–400 mesh (Merck) pretreated with 1% triethylamine in the elution solvent. ^1H and ^{13}C NMR spectra were recorded either on a Bruker WP-200 SY, on a Bruker AM 400 or on a Bruker ARX 500 spectrometer. The chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane. The letters s, d, t and m indicate the multiplicity of the signals: singlet, doublet, triplet and multiplet, respectively. The coupling constants (J) are given in hertz (Hz). The mass spectra were recorded on a LKB 9000S apparatus by electronic impact (EI, 70 eV). Spectrophotometric measures were performed on an Uvikon 933 spectrophotometer (Kontron instruments). The molar extinction coefficients (ε) are expressed in $M^{-1}\,cm^{-1}$. The melting points (mp) were determined with a Reichert hot stage microscope and were not corrected. Elemental analyses were performed by the Strasbourg analytical service of the CNRS.

Chemicals. Unless otherwise stated, all reagents were obtained from commercial suppliers and were used without purification. Anhydrous tetrahydrofuran (THF) was freshly distilled before use from sodium benzophenone ketyl.

*Synthesis of (E)-4-[2-(methylsulfanyl)-vinyl]-pyridine (**1a**) and (Z)-4-[2-(methyl-sulfanyl)-vinyl]-pyridine (**2a**).* The two isomers were synthesized, as described, from isonicotinaldehyde and methylthiomethyl-triphenylphosphonium chloride [9].

*Synthesis of 4-[1,3]-dithiolan-2-ylidenemethyl-pyridine (**3a**).* A 1.1 M solution of butyl lithium in hexane (10.8 mL; 11.9 mmol) was added to a solution of di-isopropylamine (1.65 mL; 11.9 mmol) in dry THF (19 mL) at −65 °C. The mixture was stirred for 20 min and the temperature kept below −20 °C. 4-Methylpyridine (0.525 mL; 5.4 mmol) was added, and the resulting red solution was stirred at −20 °C for 1 h. CS_2 (0.711 mL; 11.9 mmol) was then added dropwise during 10 min at −20 °C. The resulting suspension was stirred for 1 h. After addition of dibromoethane (1 mL; 11.9 mmol) the mixture was stirred at room temperature for 4 h. Water saturated Et_2O (3 mL) was added cautiously, and the solvents were removed. The residue was dissolved in $CHCl_3$ (3 mL) and the solution was washed successively with 0.5 M H_2SO_4 (3 mL), H_2O (3 mL), and brine (3 mL). The aqueous layer was basified with 4 M NaOH and extracted with $CHCl_3$ (3 mL). The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated. Compound **3a** was purified by flash chromatography on silica (eluent: ethyl acetate) and was obtained as a yellow powder (650 mg; yield 65%). mp 91–93 °C. ^1H NMR ($CDCl_3$; 200 MHz) δ: 8.51 (2H, dd, $J_1 = 4.7$, $J_2 = 1.7$); 7.27 (2H, dd, $J_1 = 4.7$, $J_2 = 1.7$); 6.55 (1H, s); 3.64–3.58 (2H, m); 3.41–3.35 (2H, m). ^{13}C NMR ($CDCl_3$; 50 MHz) δ: 149.8; 145.7; 144.2; 121.5; 113.1; 40.36; 35.9. UV(CH_3OH) λ_{max} 325 nm (ε 24,800), λ_{max} 262 nm

(ε 3400); UV(100 mM potassium phosphate buffer, pH 8.0) λ_{max} 324 nm (ε 14,200), λ_{max} 264 nm (ε 9700). MS (EI) 195, 167, 135. *Anal.* Calcd for $C_9H_9NS_2$: C, 55.35; H, 4.64; N, 7.17. Found: C, 55.46; H, 4.56; N, 6.89.

*Synthesis of 4-(2,2-bis-methylsulfanyl-vinyl)-pyridine (**4a**).* Compound **4a** was prepared essentially as described above for compound **3a**. Iodomethane (0.84 mL; 13.5 mmol) was added instead of dibromoethane. The compound was purified by flash chromatography on silica gel (eluent: ether/hexane; 1:1) and was obtained as a red oil (698 mg; yield 66%). ^{1}H NMR ($CDCl_3$; 200 MHz) δ: 8.57 (2H, m); 7.48 (2H, dd, $J_{3-2} = 4.8$, $J_{3-6} = 1.5$); 6.51 (1H, s); 2.47 (3H, s); 2.44 (3H, s). ^{13}C NMR ($CDCl_3$; 50 MHz) δ: 149.7; 143.6; 142.18; 124.3; 123.18; 40.36; 17.3. UV(CH_3OH) λ_{max} 320 nm (ε 13,250), λ_{max} 262 nm (ε 3400); UV(100 mM potassium phosphate buffer, pH 8.0) λ_{max} 316 nm (ε 11,800), λ_{max} 264 nm (ε 9700). MS (EI) 197; 182; 167; 150; 135.

*Synthesis of ethyl N-(1-pyridin-4-yl-ethylidene)-hydrazinecarboxylate (**6**).* A mixture of 4-acetylpyridine (0.55 mL; 5 mmol), carbethoxyhydrazine (521 mg; 5 mmol), methanol (4 mL), and acetic acid (12.5 μL) was refluxed 1 h and left overnight at room temperature. Compound **6** was precipitated by addition of ether and obtained in a 80% yield (831 mg) as a white powder. mp 181–183 °C. ^{1}H NMR ($CDCl_3$; 200 MHz) δ: 8.64 (2H, dd, $J_1 = 4.6$, $J_2 = 1.7$); 7.98 (1H, s); 7.64 (2H, dd, $J_1 = 4.6$, $J_2 = 1.7$); 4.34 (2H, q); 2.20 (3H, s); 1.37 (3H, t). ^{13}C NMR ($CDCl_3$; 50 MHz) δ: 154.0; 150.1; 145.3; 145.1; 120.36; 62.33; 14.52; 12.22. UV(CH_3OH) λ_{max} 276 nm (ε 15,900). MS (EI) 207; 162; 134; 119; 106. *Anal.* Calcd for $C_{10}H_{13}N_3O_2$: C, 57.96; H, 6.32; N, 20.28. Found: C, 58.10; H, 6.25; N, 20.03.

*Synthesis of 4-[1,2,3]thiadiazol-4-yl-pyridine (**7**).* Compound **6** (621 mg; 3 mmol) was added to cold thionyl chloride (1.5 mL) at 0 °C. The mixture was heated at 60 °C for 1 h, then excess thionyl chloride was removed in vacuo. Aqueous sodium carbonate was added to the residue and the aqueous layer extracted 3-fold with ethyl acetate. The organic layers were dried over Na_2SO_4, and the solvent was removed. Compound **7** was purified by flash chromatography (eluent: ethyl acetate). It was obtained as white crystals (431 mg; yield 88%). mp 119–120 °C. ^{1}H NMR ($CDCl_3$; 200 MHz) δ: 8.87 (1H, s); 8.77 (2H, dd, $J_{2-3} = 4.4$, $J_{2-5} = 1.6$); 7.94 (2H, dd, $J_{3-2} = 4.4$, $J_{3-6} = 1.6$). ^{13}C NMR ($CDCl_3$; 50 MHz) δ: 149.8; 145.7; 144.2; 121.5; 113.1; 40.36; 35.9. UV(CH_3OH) λ_{max} 270 nm (ε 6350), λ_{max} 244 nm (ε 12,800). MS (EI) 163; 135. *Anal.* Calcd for $C_7H_5N_3S$: C, 51.52; H, 3.09; N, 25.75. Found: C, 51.56; H, 2.86; N, 25.85.

*Synthesis of 4-(methylsulfanylethynyl)-pyridine (**5a**).* A 1.4 M phenyl lithium solution in benzene/ether (7:3) (0.7 mL, 1.0 mmol) was added dropwise on a stirred and precooled (−78 °C) suspension of compound **7** (150 mg; 0.92 mmol) in anhydrous THF (1.4 mL) at a such rate that the temperature did not exceed −60 °C. The reaction medium was stirred at −60 °C for further 30 min. Dimethyl sulfate (94 μL; 1 mmol) was added in one portion. The reaction was allowed to warm to 0 °C. Ice-water (5 mL) was added. The organic layer was separated and the aqueous layer extracted with ether (2 × 10 mL). The combined organic layers were dried (Na_2SO_4), and the solvents removed in vacuo. Pyridine derivative **5a** was purified by flash chromatography (eluent: ether/hexane 1:1) and was obtained as a colorless oil (112 mg;

yield 82%). ^{1}H NMR ($CDCl_3$; 200 MHz) δ: 8.51 (2H, dd, $J_1 = 4.5$, $J_2 = 1.6$); 7.20 (2H, dd, $J_1 = 4.5$, $J_2 = 1.6$); 2.50 (3H, s). ^{13}C NMR ($CDCl_3$; 50 MHz) δ: 149.67; 131.54; 124.65; 90.03; 87.82; 19.3. UV(100 mM potassium phosphate buffer, pH 8.0) λ_{max} 284 nm (ε 17,500). MS (EI) 149; 134.

*Determination of the pK_a of compounds **1a–5a***. Pyridine derivatives **1a**–**5a** (30 μM) were dissolved in a 100 mM potassium phosphate buffer at pH values between 3.0 and 8.0 (15–20 points were undertaken for each compound). UV/visible spectra were recorded between 300 and 500 nm to determine the absorbance of the pyridinium form. The absorbance values were plotted against the pH and the pK_a was taken at the inflexion point of the graph.

Synthesis of (E) 1-methyl-4(2-methylsulfanyl-vinyl)pyridinium iodide. Compound **1a** was methylated as described by Holler et al. [9]. The ^{1}H NMR spectrum of (*E*) 1-methyl-4(2-methylsulfanyl-vinyl)pyridinium iodide was taken in a 10 mM deuterated potassium phosphate buffer. ^{1}H NMR (200 MHz) δ: 8.25 (d, $J = 6.8$, 2H); 7.8 (d, $J = 15.4$, 1H); 7.6 (d, $J = 6.8$, 2H); 6.29 (d, $J = 15.4$, 1H); 4.09 (s, 3H); 2.44 (s, 3H).

Solubilization of pig brain NADase. Pig brain NADase was solubilized from an acetone powder, purchased from Sigma Chemical, according to the procedure of Windmueller and Kaplan [13]. Acetone powder (111 mg) was washed 2-fold with water to eliminate soluble contaminants. The powder was suspended in water (2 mL) under constant stirring for 10 min. The suspension was centrifuged at 20,000 rpm for 30 min with a Sigma 3K30 centrifuge. The supernatant was discarded. The pellet was suspended in a 100 mM potassium phosphate, pH 7.2 buffer (3 mL) under stirring at 37 °C. Trypsin (0.5 mg) was added to the suspension. After incubation for 40 min, the proteolysis was stopped by addition of a 0.01 M phenylmethylsulfonyl fluoride solution in isopropanol (30 μL). After 5 min at 37 °C, the suspension was chilled in an ice-bath and stirred for 30 min, and then centrifuged at 20,000 rpm for 60 min. The supernatant containing soluble NADase was used for the kinetic studies.

The concentration of protein was determined according to the Bradford method using bovine serum albumin as standard [14].

Determination of the rate of NAD^+ hydrolysis for pig brain NADase with KCN. The hydrolysis (1 mL reaction volume) was performed at 25 °C in a 100 mM potassium phosphate buffer, pH 8.0 with NAD^+ (0.45 mM) and NADase (32 μg). Aliquots (100 μL) were withdrawn at different times and added to a 1 M potassium cyanide solution (900 μL). Absorbance at 327 nm was measured to determine the amount of unhydrolyzed NAD^+ using a molar extinction coefficient of 5900 $M^{-1}cm^{-1}$ for the NAD–cyanide adduct [15]. The K_m of NAD^+ and the V_{max} were determined by varying the concentration of NAD^+ from 11 to 84 μM.

*Determination of the kinetic constants of the transglycosidation reaction with compounds **1a–5a** for pig brain NADase.* Assays were carried out in a 100 mM potassium phosphate buffer, pH 8.0 at 25 °C (final volume 1 mL). The pyridine derivatives were dissolved in acetone and concentrated stock solutions in buffer were prepared. These solutions were diluted to the appropriate concentration with buffer. The concentration of acetone was maintained constant in the assays (<5% in volume). NADase from pig brain (38 μg) was added to initiate the enzymatic reaction. Except for

compounds **1a** and **2a** ($\lambda = 380$ nm, $\varepsilon = 21{,}500\ M^{-1}\,cm^{-1}$), the transglycosidation reaction was followed by monitoring the increase of the absorbance at the λ_{max} of the corresponding NAD^+ analogs during 10 min (Table 1).

The K_m of NAD^+ was determined at a fixed concentration of compound **1a** (3 mM) and the concentration of NAD^+ was varied from 0.015 to 0.45 mM. The kinetic constants of compounds **1a**–**5a** were established at a fixed concentration of NAD^+ (0.45 mM). The concentration of pyridine derivatives was varied from 0.045 to 1.35 mM for compound **1a**, from 0.090 to 0.9 mM for compound **2a**, from 0.13 to 1.3 mM for compound **3a**, from 0.45 to 2.25 mM for compound **4a**, and from 0.62 to 3.1 mM for compound **5a**. The reactions were initiated by the addition of NADase (26–95 μg depending on the substrate).

*Preparation of the NAD^+ analogs **1b**–**5b***. They were synthesized enzymatically. The same procedure was used for each analog. The preparation of analog **1b** is given. Pyridine derivative **1a** (80 mg) and NAD^+ (80 mg) were incubated in a 10 mM Tris/HCl buffer, pH 8.0 (final volume 80 mL) with NADase from pig brain (20 mg). The reaction was followed by measuring the absorbance at 374 nm. The reaction was stopped when the absorbance reached a plateau. The excess of compound **1a** was extracted with ethyl acetate. The aqueous layer was applied on a Dowex 1×2 ($HCOO^-$ form) column (1×5 cm) equilibrated with water. The column was washed with water to eliminate residual compound **1a**. A gradient of formic acid (0–0.4 M) (2×50 mL) was applied to elute the retained compounds. The analog was detected by its absorbance at 374 nm. Fractions having the highest 374 nm/252 nm absorbance ratio were combined and lyophilized.

The spectrophotometric properties of the different NAD^+ analogs are given in Table 1. The yields of the isolated analogs were about 40–60%. NMR data of the NAD^+ analogs **1b**–**5b** are given below.

Analogs **1b** and **2b**. ^1H NMR (D_2O; 400 MHz) δ: 8.73 (2H, d, $J = 7$; *Z* form); 8.65 (2H, d, $J = 7$; *E* form); 8.43 (1H, s, *E* and *Z* forms); 8.21 (1H, s, *E* and *Z* forms); 7.89 (1H, d, $J = 15$, *E* form); 7.78 (2H, d, $J = 7$, *Z* form); 7.58 (2H, d, $J = 7.5$, *E* form); 7.4 (1H, d, $J = 11$, *Z* form); 6.39 (1H, d, $J = 11$, *Z* form); 6.18 (1H, d, $J = 15$, *E* form); 6.11 (1H, d, $J = 6.5$, *E* form); 6.09 (1H, d, $J = 6$, *Z* form); 5.98

Table 1
Spectrophotometric properties of the NAD^+ analogs **1b**–**5b**

Compounds	λ_{max} (nm)	ε ($M^{-1}\,cm^{-1}$)	λ_{max} (nm)	ε ($M^{-1}\,cm^{-1}$)
1b	252[a]	13,500[a]	374[a]	22,300[a]
2b	—[b]	—[b]	—[b]	—[b]
3b	254	13,300	405	26,500
4b	255	14,700	405	23,500
5b	259	14,500	354	17,300

Note. The NAD^+ analogs were dissolved in 100 mM potassium phosphate buffer, pH 8.0. The UV/visible spectra were recorded between 240 and 480 nm. Measures were carried out in triplicate.

[a] For a mixture of 90% **1b** and 10% **2b** due to the isomerization of the methylsulfanyl-vinyl group.

[b] Could not be determined due to the isomerization of **2b** to **1b**.

(1H, d, $J = 5$, *E* and *Z* forms); 4.5-3 (m, *E* and *Z* forms); 2.72 (3H, s, *Z* form); 2.56 (3H, s, *E* form). ^{13}C NMR (D_2O; 100.6 MHz) δ: 153.6; 150.4; 148.4; 146.6; 145.9; 142.2; 139.5; 122.0; 118.4; 117.4; 98.6; 87.7; 86.4; 84.4; 77.3; 74.6; 70.7; 70.6; 65.5; 65.1; 13.6.

Analog **3b**. ^{1}H NMR (D_2O; 400 MHz) δ: 8.35 (2H, d, $J = 7.1$); 8.21 (1H, s); 8.06 (1H, s); 7.34 (2H, d, $J = 7.1$); 6.30 (1H, s); 5. 85 (1H, d, $J = 6$); 5.67 (1H, d, $J = 5$); 3.61 (2H, m); 3.33 (2H, m). ^{13}C NMR (D_2O; 100.6 MHz) δ: 154.9; 152.6; 151.9; 149.6; 139.2; 138.4; 122.4; 118.0; 109.2; 100.2; 97.4; 84.3; 83.8; 76.9; 73.9; 70.3; 69.1; 68.3; 65.4; 65.0; 41.5; 36.1.

Analog **4b**. ^{1}H NMR (D_2O; 400 MHz) δ: 8.61 (2H, d, $J = 6.8$); 8.39 (2H, d, $J = 6.8$); 8.18 (1H, s); 8.04 (1H, s); 5. 83 (2H, m); 5.69 (1H, m); 4.4–4 (m); 2.43 (3H, s); 2.35 (3H, s). ^{13}C NMR (D_2O; 100.6 MHz) δ: 152.8; 151.5, 149.5; 148.6; 140.7; 138.8; 123.9; 119.0; 113.1; 102.4; 98.5; 87.0; 86.3; 84.3; 77.2; 74.4; 70.7; 70.6; 65.7; 65.3; 16.7; 15.7.

Analog **5b**. ^{1}H NMR (D_2O; 400 MHz) δ: 8.75 (2H, d, $J = 6.8$); 8.47 (1H, s); 8.28 (1H, s); 7.67 (2H, d, $J = 6.8$); 6.04 (1H, d, $J = 5.6$); 5.89 (1H, d, $J = 5.2$); 2.51 (3H, s). ^{13}C NMR (D_2O; 100.6 MHz) δ: 151.3; 149.2; 147.1; 141.6; 141.4 139.4; 139.4; 127.1; 118.3; 105.1 98.9; 90.5; 87.4; 86.4; 84.0; 77.4; 70.6; 70.3; 65.2; 64.9; 18.8.

*Isomerization of NAD$^+$ analogs (**1b**) and (**2b**)*. The transglycosidation reaction with compounds **1a** and **2a** were followed by ^{1}H NMR (500 MHz). The pyridine derivative **1a** or **2a** (1 mg), NAD$^+$ (1.5 mg) and NADase from pig brain (3 mg) were incubated at 37 °C in a 10 mM deuterated potassium phosphate buffer (final volume 500 μL). The deuterated buffer was obtained from a 100 mM potassium phosphate buffer, pH 8.0 (10 mL) by five successive "lyophilization/dissolution in D_2O" steps. Finally the residue was dissolved in 10 mL of D_2O to obtain a stock solution of 100 mM deuterated potassium phosphate buffer. NADase was dialyzed against 10 mM deuterated buffer by a series of concentration/dilution steps by ultracentrifugation using a Centricon YM-10 unit (Millipore). The pyridine derivatives were dissolved in a minimum of deuterated acetone (20 μL). ^{1}H NMR spectra were recorded after 7, 60, 120 and 240 min and 24 h incubation. The signal of H_2O was suppressed by presaturation. The ratio of the products resulting from the enzymatic reaction was determined by integration of typical signals: for NAD$^+$ δ: 9.45 (1H, s, N2); 9.28 (1H, d, $J = 6.5$, N6); 8.95 (1H, dt, $J_{4-5} = 8$, $J_{4-2} = 3$, $J_{4-6} = 1.5$, N4); 8.54 (1H, s, A8); 8.31 (1H, dd, $J_{5-4} = 8$, $J_{5-6} = 6.5$, N5); 8.26 (1H,s, A2); 6.20 (1H, d, $J = 5.5$, N′1); 6.14 (1H, d, $J = 6$, A′1) [16]; for free nicotinamide $\delta = 9.03$ (1H, m, N2); 8.08 (1H, m, N6) [17], anomeric protons for α- and β-ADP–ribose $\delta = 5.31$ (1H, d, $J = 2.5$) and $\delta = 5.43$ (1H, d, $J = 4$), respectively [18], vinylic protons for NAD$^+$ analogs **1b** (δ: 6.18 and 7.89) and **2b** (δ: 6.39 and 7.4).

*Hydrolysis of NAD$^+$ analogs **1b** and **5b** with pig brain NADase*. The analogs (40 μM) were incubated at 25 °C in a 0.1 M sodium phosphate buffer, pH 8.0 in the presence of pig brain NADase (50 μg). The enzymatic reaction was followed by monitoring the absorbance at 374 and 354 nm for compounds **1b** and **5b**, respectively, during 30 min.

Kinetic studies with ADP-ribosyl cyclase from A. californica. ADP-ribosyl cyclase from *A. californica* was obtained from Dr. F. Schuber [2]. Samples were also

purchased from Sigma. Except otherwise indicated the enzymatic reactions were performed in a 100 mM potassium phosphate buffer, pH 8.0 at 25 °C.

The rate of hydrolysis of NAD^+ was measured according to Kaplan [15]. The reaction medium (600 L) contained NAD^+ (0.45 mM) and ADP-ribosyl cyclase (0.37 μg). Aliquots of 100 μL were withdrawn at fixed time to determine the concentration of unhydrolyzed substrate.

The kinetic constants for the transglycosidation reaction with pyridines **1a**–**5a** were determined as described above. The reactions were performed at saturating concentration of NAD^+: 1 mM for assays with compounds **1a** and **2a**, 0.6 mM for assays with pyridine **3a**, 2 mM for assays with compounds **4a** and **5a**. The concentration of pyridine derivatives was varied from 80 to 500 μM for compound **1a**, from 8 to 65 μM for compound **2a**, from 14 to 210 μM for compound **3a**, from 80 to 560 μM for compound **4a**, from 190 to 1900 μM for compound **5a**. The reactions were initiated by the addition of enzyme (0.2 μg) and monitored during 2 min.

3. Results

*3.1. Syntheses of pyridine derivatives (**1a**–**5a**)*

Compounds **1a** and **2a** were prepared according to Holler et al. [9]. The two isomers were easily distinguished by the ^1H NMR coupling constants of the vinylic protons: 15.5 Hz for the (*E*) isomer and 11 Hz for the (*Z*) isomer.

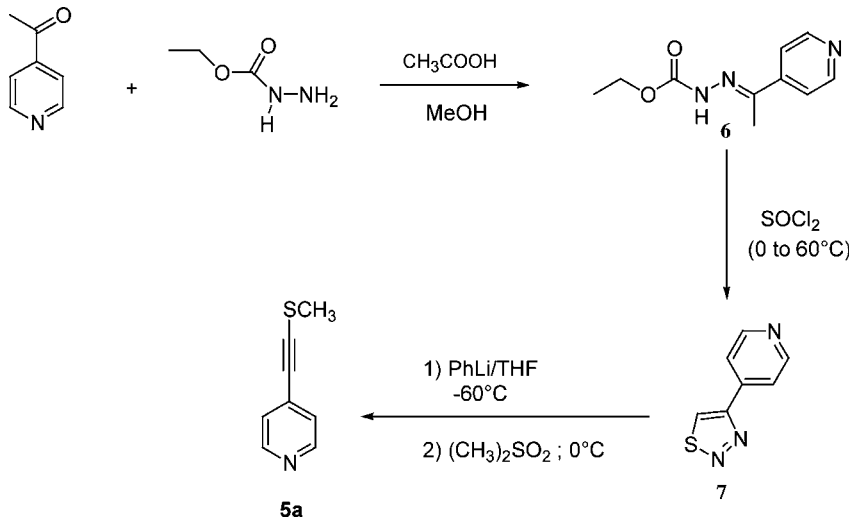
The method described by Goodwin et al. [19] for the preparation of pyridines substituted at position 2 with ketene dithioacetal groups was applied to synthesize compounds **3a** and **4a**. Compound **4a** was unstable and thus, prepared and purified before use.

The synthesis of 1-alkynylthioethers via 1,2,3-thiadiazole by Raap and Micetich [20] was efficiently extended to the preparation of pyridine **5a**. The compound was obtained in 3 steps from 4-acetyl-pyridine (Scheme 3).

The pK_a of the pyridine derivatives **1a**–**5a** was measured to determine the optimal pH for the transglycosidation reaction. As the enzymatic substrate is the unprotonated pyridine, the enzymatic assays were performed at 2 pH units above the pK_a in order to have high concentration of unprotonated pyridine. The protonated and unprotonated forms absorbing at different wavelengths, their pK_a's were determined spectrophotometrically (Table 2). The pK_a values of the pyridine nitrogen group were significantly higher than that of nicotinamide ($pK_a = 3.33$) or pyridine ($pK_a = 5.23$) [11], due to the electron donating effect of the alkylsulfanyl-vinyl group.

*3.2. Transglycosidation reaction with pyridine derivatives **1a**–**5a** catalyzed by pig brain NADase: structure of the transglycosidation products*

The reaction with pyridine derivatives **1a** and **2a** was extensively studied. The incubation of both compounds with NAD^+ and NADase led to the formation of an absorption band in the visible region, suggesting that the enzyme exchanged



Scheme 3. Synthesis of 4-(methylsulfanylethynyl)pyridine **5a**.

Table 2
pK_a of compounds **1a**–**5a**

Compounds	λ_{max} Unprotonated form	λ_{max} Protonated form	pK_a
1a	304	355	6.0
2a	310	358	5.8
3a	325	377	6.5
4a	316	374	6.2
5a	284	335	4.9

Note. Pyridine derivatives **1a**–**5a** (30 μM) were dissolved in a 100 mM potassium phosphate buffer at pH between 3 and 8. Spectra were recorded between 300 and 500 nm to determine the absorbance of the pyridinium form. The absorbance values were plotted against the pH and the pK_a was taken at the inflexion point of the graph.

nicotinamide with both pyridine derivatives **1a** and **2a**. The transglycosidation reaction was performed on a large scale to confirm the structure of the NAD^+ analogs **1b** and **2b**. The spectroscopic analysis of the isolated products showed that both gave a mixture of analogs **1b** and **2b** in a same 9/1 ratio. Clearly a *cis–trans* isomerization of the methylsulfanyl-vinyl group took place in the reaction medium and/or during the purification of the analogs. Following the transglycosidation reaction by ^1H NMR solved this question (Figs. 1 and 2). Integration of typical signals allowed quantifying of the different molecules present in the reaction medium (Tables 3 and 4). The assignments of the peaks for NAD^+, free nicotinamide and ADP–ribose were based on published spectra [16–18]. The NMR spectra clearly showed that the isomerization occurred in the reaction medium. This fact was particularly perceptible with pyridine **2a** where the analogs **2b/1b** ratio decreased during the incubation time.

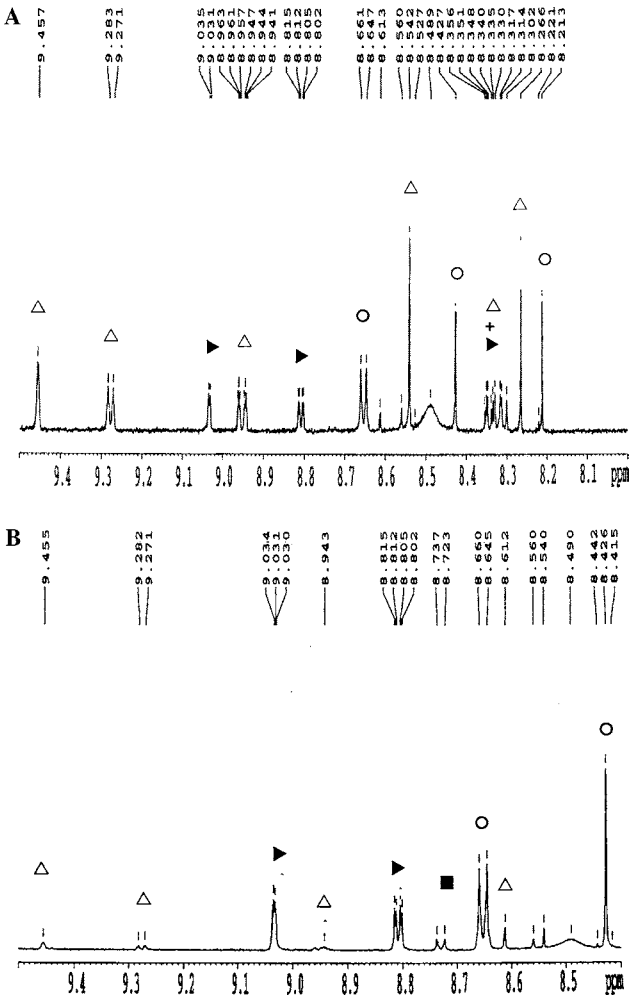


Fig. 1. Transglycosidation reaction of pig brain NADase with pyridine **1a**. Reaction conditions are given in Table 3. Partial ^{1}H NMR (500 MHz) spectra after incubation of 7 min (**A**) and 360 min (**B**) at 37 °C are shown. Characteristic peaks of NAD^+ (△), free nicotinamide (▶), NAD^+ analogs **1b** (○) and **2b** (■) are annotated.

After 24 h, the 9/1 ratio in favor of analog **1b** was reached. The stability of pyridine derivatives **1a** and **2a** in the deuterated buffer was checked at 37 °C. No isomerization was detected after an incubation of 24 h. So the formation of the mixture of NAD^+ analogs **1b** and **2b** was not due to a preliminary isomerization of the pyridine derivatives. The catalytic event did not seem to be responsible for the isomerization. Indeed the alkylation of pyridine **1a** with iodomethane gave also a mixture of (*E*) and (*Z*) isomers in the same 9/1 ratio as evidenced by ^{1}H NMR in deuterated buffer. Likely the isomerization took place after the analogs dissociated from the enzyme.

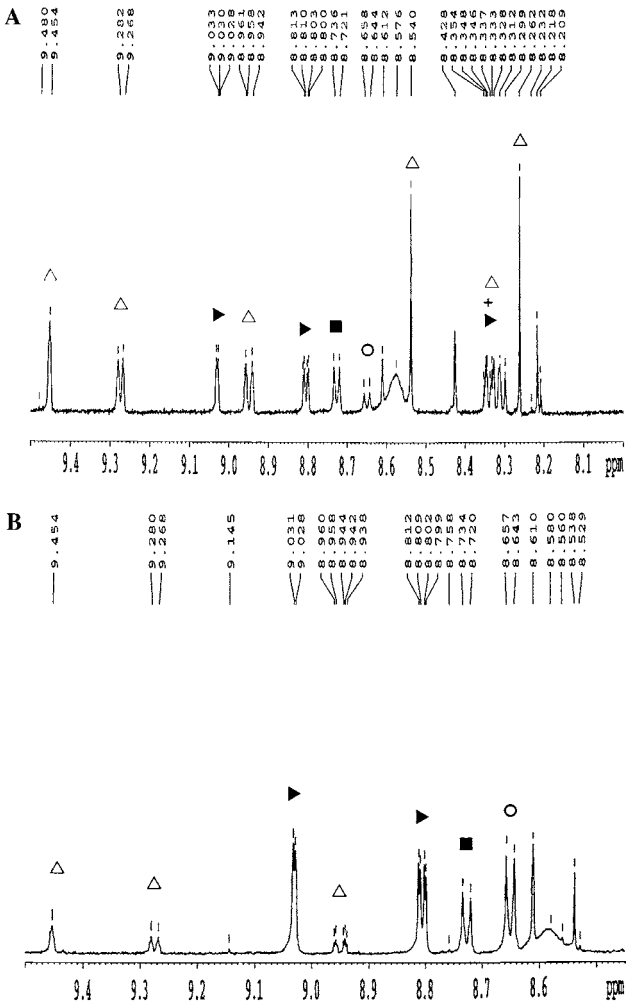


Fig. 2. Transglycosidation reaction of pig brain NADase with pyridine **2a**. Reaction conditions are given in Table 4. Partial ^{1}H NMR (500 MHz) spectra after incubation of 7 min (**A**) and 360 min (**B**) at 37 °C are shown. Characteristic peaks of NAD^{+} (△), free nicotinamide (▶), NAD^{+} analogs **1b** (○) and **2b** (■) are annotated

In the two experiments, NAD^{+} disappeared at almost the same rate. On the other hand the efficiency of pyridine exchange, illustrated by the ratio of analog/ADP–ribose concentrations, was more important with compound **1a** (87/13) than with compound **2a** (69/31). Yost and Anderson reported for NADase from *Bungarus fasciatus* venom that the efficiency of the transglycosidation increased linearly with respect to the concentration of the pyridine derivatives [12]. In our case, as the reactions were performed at the same concentration of pyridine derivative, the higher efficiency with compound **1a** could be linked to the different affinity of the enzyme for the

Table 3
^{1}H NMR study of the transglycosidation with compound **1a**

Reaction time (min)	7	60	120	240	360
Disappeared NAD^+ (%)	7	43	62	85	90
Analog **1b** (%)	Traces	89	81	81	78
Analog **2b** (%)	ND	11	6	6	9
α- and β-ADP–ribose (%)	ND	ND	13	13	13

Note. Pyridine derivative **1a** (1 mg), NAD^+ (1.5 mg) and NADase from pig brain (3 mg) were incubated in a 10 mM deuterated potassium phosphate buffer (final volume 600 μL) at 37 °C. ^{1}H NMR (500 MHz) spectra were recorded at indicated time. The proportion of disappeared NAD^+, analogs **1b** and **2b** and ADP–ribose was calculated from the integration of characteristic peaks: for NAD^+ δ: 9.45 (1H, s, N2); 9.28 (1H, d, $J = 6.5$, N6); 8.95 (1H, dt, $J_{4-5} = 8$, $J_{4-2} = 3$, $J_{4-6} = 1.5$, N4); 8.54 (1H, s, A8); 8.31 (1H, dd, $J_{5-4} = 8$, $J_{5-6} = 6.5$, N5); 8.26 (1H, s, A2); 6.20 (1H, d, $J = 5.5$, N′1); 6.14 (1H, d, $J = 6$, A′1) [16]; for free nicotinamide $\delta = 9.03$ (1H, m, N2); 8.08 (1H, m, N6) [17], anomeric protons for α- and β-ADP–ribose $\delta = 5.31$ (1H, d, $J = 2.5$) and $\delta = 5.43$ (1H, d, $J = 4$), respectively [18], vinylic protons for NAD^+ analogs **1b** (δ: 6.18 and 7.89) and **2b** (δ: 6.39 and 7.4).

ND, not detected.

Table 4
^{1}H NMR study of the transglycosidation with compound **2a**

Reaction time (min)	7	60	120	240	360
Disappeared NAD^+ (%)	6	42	57	78	87
Analog **1b**	ND	26	28	37	46
Analog **2b**	Traces	46	43	32	26
α- and β-ADP–ribose	ND	28	29	31	28

Note. Pyridine derivative **2a** (1 mg), NAD^+ (1.5 mg) and NADase from pig brain (3 mg) were incubated in a 10 mM deuterated potassium phosphate buffer (final volume 600 μL) at 37 °C. ^{1}H NMR (500 MHz) spectra were recorded at indicated time. The proportion of disappeared NAD^+, analogs **1b** and **2b** and ADP–ribose was calculated from the integration of characteristic peaks as described in Table 2.

ND, not detected.

Table 5
Kinetic constants of the transglycosidation reaction for pig brain NADase

Substrates	K_m (μM)	V_{max} (μM/min/mg prot)	V_{max}/K_m (min^{-1} mg $prot^{-1}$)
1a	300	77[a]	0.257
2a	760	45[a]	0.059
3a	1460	67	0.046
4a	950	38	0.040
5a	470	18	0.038
NAD^+	34[b]	77[b]	—

Note. The reaction conditions are given in Section 2. Except for compounds **1a** and **1b** (see text), the transglycosidation reactions were followed at the λ_{max} of the generated NAD^+ analogs (Table 4).

[a] Determined at 380 nm, $\varepsilon = 21{,}500\ M^{-1}\,cm^{-1}$ for a mixture of analogs **1b** and **2b** (9/1).

[b] Determined with compound **1a** as the fixed substrate.

two isomers. Indeed, taking the K_m values in Table 5 into account, the enzymatic reactions were performed at a concentration corresponding to 35-fold the K_m with pyridine **1a** and to 15-fold the K_m with pyridine **2a**.

Due to the fact that transglycosylation reaction with either reagent **1a** or **2a** furnished a mixture of same ratio of (*E*) and (*Z*) isomers **1b** and **2b** and that pure NAD^+ products **1b** and **2b** could not be isolated, the λ_{max} and ε spectrophotometric data of the two products could not be determined accurately. The absorption bands of the 2 isomers may have different intensity because (*E*) 1-methyl-4-[2-(methylsulfanyl)-vinyl]pyridinium iodide has an 1.5-fold higher ε than the (*Z*)-isomer [9]. Nevertheless, the ε of the isolated NAD^+ analog was determined from the 9/1 mixture of the two isomers **1b** and **2b** (Table 1).

The problem encountered with derivatives **1a** and **2a** led us to synthesize and to test compounds **3a**–**5a**. All the 3 derivatives were substrate of the NADase. The ^1H and ^{13}C NMR spectra of the isolated NAD^+ analogs **3b**–**5b** agreed with the anticipated structures.

*3.3. Transglycosidation reaction with pyridine derivatives **1a**–**5a** catalyzed by pig brain NADase: kinetic studies*

The transglycosidation reaction may satisfy several criteria to be suitable as an enzymatic assay: linearity of the absorbance increase in function of time, saturating kinetics, proportionality between the rate and enzyme concentration. Assays were performed with compound **1a**. The reaction was followed at a slightly higher wavelength (380 nm) than the λ_{max} to minimize the ground level of absorbance. So the reaction could be followed for a longer time and with high concentrations of compound **1a** without saturation of the spectrophotometer. The reaction was generally followed over 10 min to determine the rate of the enzymatic reaction, but the increase of the absorbance was linear for at least 1 h ($\Delta DO_{380} = 0.4$). In the enzymatic reaction the pyridine derivatives competed with water producing NAD^+ analogs and ADP–ribose, respectively. The linearity of the absorbance increase indicated that the product ratio NAD^+ analog/ADP–ribose was constant during the reaction time and that the enzyme remained stable. The rate of the transglycosidation was also proportional to the concentration of protein (*data not shown*).

The kinetic constants (K_m and V_{max}) of pyridines **1a**–**5a** are given in Table 5. The transglycosidation rates with pyridines **1a** and **2a** were calculated by using the molar extinction coefficient obtained with the 9/1 mixture of compounds **1b** and **2b**. However as the equilibrium was plainly in favor of analog **1b** and was reached rapidly, the transglycosidation rate for compound **1a** could reasonably be considered as close to the true rate. On the other hand, the rate calculated with this ε value for the base exchange with compound **2a** would be underestimated, the equilibrium being reached more slowly.

The maximal rate for the transglycosidation ($V_{max} = 77\,\mu M/min/mg\,prot$ with compound **1a**) was lower than the NAD^+ disappearance rate determined by the KCN method ($V_{max} = 197\,\mu M/min/mg\,prot$ with NAD^+ as the substrate).

Table 6
Kinetic constants of the transglycosidation reaction for ADP-ribosyl cyclase of *Aplysia californica*

Substrates	K_m (μM)	V_{max} (mM/min/mg prot)	V_{max}/K_m (min^{-1} mg $prot^{-1}$)
1a	110	116[a]	1055
2a	37	45[a]	1216
3a	45	78	1733
4a	58	32	552
5a	290	94	324

Note. The reaction conditions are given in Section 2. Except for compounds **1a** and **2a** (see text), the transglycosidation reactions were followed at the λ_{max} of the generated NAD^+ analogs (Table 4).

[a] Determined at 380 nm, $\varepsilon = 21{,}500\ M^{-1}\,cm^{-1}$ for a mixture of analogs **1b** and **2b** (9/1).

*3.4. Transglycosidation reaction with pyridine derivatives **1a–5a**, catalyzed by ADP-ribosyl cyclase from A. californica*

The kinetic constants of the cyclase for compounds **1a**–**5a** are given in Table 6. Pyridine **3a** seemed to be the most efficient substrate. The NAD^+ hydrolysis rate measured with the KCN method (2652 mM/min/mg prot) at saturating concentration of NAD^+ was about 34-fold higher than that of the transglycosidation with compound **3a**.

4. Discussion

The base exchange of nicotinamide with pyridine derivatives was considered as a noteworthy property of NADases to synthesize NAD^+ analogs [10]. As pointed out for the calf spleen NADase [21], the transglycosidation reaction may occur according to a ping-pong Bi Bi mechanism. After cleavage of the glycosidic nicotinamide–ribose bond and release of nicotinamide, pyridine derivatives would compete with water for the nucleophilic attack of the ADP-ribosyl oxocarbenium ion intermediate. The corresponding NAD^+ analogs are generated with retention of the β configuration [10]. All NADases do not perform this reaction. The enzymes catalyzing the transglycosidation are easily revealed by the fact that their activity is inhibited by nicotinamide [15].

Only few extensive studies of the transglycosidation reaction catalyzed by NADases are reported. The reason could be the difficulty to perform the enzymatic assays, based on the separation of the different reaction products by high performance liquid chromatography [7,12]. Yost and Anderson described some aspects on the translycosidation of venom NADase such as substrate specificity of the base exchange [11,12,22]. For CD38, radiolabelled nicotinamide was used to study the kinetic mechanism of base exchange [1]. The development of a specific assay for the transglycosidation reaction would be helpful. With our test the progress of the reaction is monitored continuously by following the absorbance increase due to the formation of the NAD^+ analogs and the rate is calculated instantaneously from the slope.

All five pyridine derivatives tested were substrate of the enzyme in the transglycosidation reaction. The pyridine derivatives had relatively high pK_a values (Table 2). Therefore, as pointed out by Tarnus and Schuber [23] which found a 6000-fold lower hydrolysis rate for a NAD^+ analog with a pyridine derivative having a pK_a of nearly 6.0 with regard to NAD^+, the hydrolysis of the NAD^+ analogs by pig brain NADase was insignificant. The high pK_a values required performing the reactions at pH 8, slightly higher than the usual, pH 7.2. The enzyme has a broad pH optimum (pH 5 to 8.5) and the influence on the rate would be negligible (<10%) [13].

The isomerization of NAD^+ analogs **1b** and **2b** pointed out by the NMR analysis of the enzymatic reaction could be attributed to the push–pull nature of the double bond. As reported for the (*Z*) to (*E*) isomerization of (*Z*) 1-methyl-4-[2-(methylsulfanyl)-vinyl]pyridinium iodide, the electron transfer from the electron donating methylsulfur group to the electron withdrawing pyridinium group lowers the isomerization barrier [24]. While the isomerization in DMSO-d6 of the (*Z*) 1-methyl-4-[2-(methylsulfanyl)-vinyl]pyridinium iodide to the (*E*) isomer was rapid and quantitative, the isomerization of the analogs **1b** and **2b** in the buffered medium was slower and gave a 9/1 mixture in favor of analog **1b**. The nature of the solvent and of the N-substituent (a ribofuranosyl group instead of a methyl group) might be at the origin of these differences.

The observed saturating kinetics confirmed that a specific ternary complex between the enzyme, the ADP-ribosyl oxocarbenium ion intermediate and the pyridine derivative takes place before the nucleophilic attack. The transglycosidation reaction did not result from a bimolecular reaction between the ADP-ribosyl oxocarbenium ion intermediate and the pyridine derivative. With pig brain NADase the V_{max}/K_m ratio indicates that compound **1a** was the most efficient (Table 5). Compound **1a** had the lowest K_m, about 2- to 3-fold lower than its isomer **2a**. Any of the pyridines **3a**–**5a** synthesized to avoid the problem of the *cis–trans* isomerization of the methylsulfanyl-vinyl group, can be used as an alternative. However compounds **3a** and **4a** were poorly soluble in buffer and had high K_m values. So they were not suitable for kinetic studies because the enzymatic reaction could not be performed in optimal conditions of substrate concentration. The transglycosidation rate with compound **5a** was 4-fold slower than the exchange with compound **1a**.

The transglycosidation assay was applied to ADP-ribosyl cyclase from *A. californica*. This multifunctional enzyme catalyzes not only the formation of cyclic-ADP–ribose but also the exchange of nicotinamide of $NAD(P)^+$ with nicotinic acid [7]. As cyclic ADP–ribose, the $NADP^+$ analog, nicotinic acid adenine dinucleotide phosphate ($NAADP^+$) is implicated in the Ca^{2+} release mechanism while no activity for the NAD^+ analog $NAAD^+$ has been detected [7,8]. The base exchange reaction is dominant over the cyclization at acidic pH. At pH 7.0 with NAD^+ as the substrate, even in the presence of nicotinic acid, only the synthesis of cyclic ADP–ribose is observed [7]. With compounds **1a**–**5a** the transglycosidation reaction occurred even at pH 8.0. Our assay would make it possible to determine the factors privileging one reaction or the other and to show if the base exchange between nicotinamide and nicotinic acid is likely to occur in vivo and is not an in vitro reaction.

The comparison of the kinetic parameters of pig brain NADase and ADP-ribosyl cyclase showed some differences with regard to their affinity for the pyridine derivatives. The K_m values of the cyclase were lower than those of the NADase and the two enzymes showed a different specificity for the pyridine derivatives. If the NADase seemed to better recognize compounds **1a** and **5a**, the cyclase preferred compounds **2a**–**4a**. This reflects a different nicotinamide binding site, the recognition site for the pyridine derivatives.

The hydrolysis of NAD^+ (measured with the KCN method) was faster (about 2.5- and 34-fold for pig brain NADase and ADP-ribosyl cyclase, respectively) than the transglycosidation. As nicotinamide, the pyridine derivatives would inhibit the NAD^+ cleavage. In the case of calf spleen NADase, a 3-fold decrease of the reaction rate was observed in the presence of nicotinamide. The base exchange with [^{14}C]nicotinamide was about 10-fold slower than the NAD^+ cleavage [21].

5. Conclusion

We developed a continuous spectrophotometric assay allowing the determination of the transglycosidation reaction of NADases. The assay is very sensitive owing to the high absorption coefficient of the NAD^+ analogs. More the latter absorb in the near visible so that the measurements would not be hindered by the absorption of proteins and $NAD(P)^+$. Optimal assay conditions have been determined for the two enzymes and are the following: $[NAD^+] = 0.45\,mM$ and [compound **1a**] $= 3\,mM$ for the pig brain NADase; $[NAD^+] = 0.6\,mM$ and [compound **3a**] $= 1\,mM$ for the ADP-ribosyl cyclase. The use of our assay as a common method to measure the enzymatic of NADases could also be envisaged.

Acknowledgments

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