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SUPPORTING INFORMATION

A Novel Fluorescent Probe for NAD-Consuming Enzymes

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(i) GENERAL METHODS

All reagents were obtained commercially and used as received, including anhydrous solvents over molecular sieves, unless otherwise stated. For the preparation of dinucleotides, $MnCl_2$ solution in formamide was dried over molecular sieves (4Å) and stored under nitrogen atmosphere. All enzymes were purchased from Sigma. TLC was performed on precoated slides of Silica Gel 60 F_{254} (Merck). Spots were visualised under UV light (254/365nm). Reaction products were characterised by high-resolution mass spectrometry (HR-MS), ^{1}H, ^{13}C and ^{31}P NMR spectroscopy, and HPLC. ^{1}H NMR spectra were recorded at 298K on a Varian VXR 400 S spectrometer at 400 MHz or on a Bruker Avance DPX-300 spectrometer at 300MHz. ^{13}C NMR spectra were recorded at 300K on a Bruker BioSpin GmbH at 150.90MHz. ^{31}P NMR spectra were recorded at 298K on a Varian VXR 400 S spectrometer at 161.98MHz. Chemical shifts (δ) are reported in ppm (parts per million) and coupling constants (J) in Hz. Accurate electrospray ionization mass spectra were obtained on a Finnigan MAT 900 XLT mass spectrometer at the EPSRC National Mass Spectrometry Service Centre, Swansea.

Ion-pair and ion-exchange chromatography was performed on a Biologic LP chromatography system equipped with a peristaltic pump and a 254nm UV Optics Module. For detailed conditions, see below. Analytical chromatography (HPLC) was carried out on a Perkin Elmer series 200 equipped with a Supelcosil LC-18T column (25cm x 4.6mm, particle size 5µm), a diode array detector (detection wavelengths: 254 and 280nm) and a column oven (temperature: 30 ºC). For detailed conditions, see below. Absorbance spectra were recorded on a PerkinElmer Lambda 25 UV-Vis spectrometer at room temperature in FarUV quartz cells (path length 1.0cm). Fluorescence spectra were recorded on a PerkinElmer LS-45 spectrometer at room temperature in a quartz micro fluorescence cell (path length 1.0cm). Fluorescence assays were performed in NUNC 96 plates on a BMG labtech PolarStar microplate reader equipped with an absorbance filter at 300 ± 5 nm and with an emission filter at 410 ±5 nm (gain 15%). Fluorescence quantum yields (Φ) of NAD and AMP derivatives (**3a-e** and **5a-e**) were determined by the comparative method reported in the literature (A. T. R. Williams, S. A. Winfield, J. N. Miller, *Analyst* **1983**, *108*, 1067), using 2-aminopyridine in 0.1M H_2SO_4 (Φ=0.60) and tryptophan in water, pH 7.2 (Φ=0.14) as reference standards.

CHROMATOGRAPHY CONDITIONS

Preparative chromatography – purification method 1: Ion-pair chromatography was performed using Lichroprep RP-18 resin and a variable gradient of methanol against 0.05M TEAB (triethylammonium bicarbonate buffer). Product containing fractions were combined and reduced to dryness. The residue was co-evaporated repeatedly with methanol to remove residual TEAB.

Preparative chromatography – purification method 2: Anion exchange chromatography was performed using Bioscale™ Mini Macro-Prep High Q cartridges and a gradient of 0–100% 1M TEAB (pH 7.3) against MilliQ H_2O. Product containing fractions were combined and reduced to dryness. The residue was co-evaporated repeatedly with methanol to remove residual TEAB.

Analytical chromatography (HPLC): Samples (injection volume: 20µL) were analysed using a mobile phase composed of 0.05M phosphate buffer at pH 8 (solvent A) and methanol (solvent B), according to the following gradient steps (flow rate: 1.5mL/min):

0 to 2 min	90% A-10% B
2 to 17 min	10-50% B
17 to 19 min	50% B
19 to 21 min	50-10% B
21 to 25 min	90% A-10% B

(ii) PREPARATION OF NAD DERIVATIVES **3a-e**

General synthetic method (i): *Synthesis of 8-substituted AMP derivatives* **5a-e** *by Suzuki-Miyaura cross-coupling* [1]. 8-Br-AMP **4**, Na_2PdCl_4, TPPTS, K_2CO_3 and (hetero)aryl boronic acid were placed in a flask and purged with nitrogen for 15mins. Degassed water was added by syringe to the reaction vessel and the mixture was stirred at the appropriate temperature under nitrogen for the given time. When the reaction was complete, the mixture was evaporated *in vacuo*, and the crude purified by chromatography, as indicated below for each compound.

General synthetic method (ii): *Synthesis of AMP morpholidates* **6a-e** [2]. The respective 8-substituted AMP derivative **5** (1 equiv.) was dissolved in DMSO, and then co-evaporated with DMF (3x). The residue was dissolved in dry DMSO and morpholine (6 equiv.) was added. After 5 mins, 2,2'-dithiopyridine (3 equiv.) was added and, subsequently, triphenylphosphine (3 equiv.). The yellow solution was stirred at room temperature under a nitrogen atmosphere until TLC showed complete conversion. NaI in acetone (0.1M) was added drop-wise to the reaction mixture until a solid precipitate formed. The precipitate was washed repeatedly with acetone until it was colourless. The colorless crude was dried and used in the next reaction step without further purification.

General synthetic method (iii): *Synthesis of dinucleotides* **3a-e** [3]. The respective AMP morpholidate **6** (1.2 equiv.) and β-NMN (1 equiv.) were co-evaporated with pyridine (3x). The flask with the residual solid was purged with nitrogen for 15mins. Anhydrous $MgSO_4$ (2 equiv.) and $MnCl_2$ (0.2M in formamide, 1.5 equiv.) were added and the reaction was stirred at room temperature under nitrogen until TLC showed complete conversion. The reaction was stopped by dropwise addition of MeCN until a white precipitate formed. The supernatant was removed and the solid dissolved in water and evaporated to dryness *in vacuo*. The crude was purified by chromatography, as indicated below for each compound.

8-bromo adenosine monophosphate (4) [4]. To a solution of adenosine monophosphate (0.5g, 1 equiv.) in aqueous KH_2PO_4 (0.25M, pH 5, 100mL), neat bromine (0.5mL, 6.7 equiv.) was added dropwise. The reaction was stirred at room temperature in the dark overnight. The aqueous solution was washed with DCM until the colour remained pale yellow, and evaporated to dryness. The crude residue was purified by purification method 1 (0-10% MeOH against 0.05M TEAB buffer over 400mL, flow: 5mL/min, fraction size: 5mL). The title compound was isolated as a colorless oil (0.56g, 2.2 equiv. TEA, 60% yield). ^{1}H NMR (400MHz, D_2O) δ: 8.03 (1H, s, H-2), 5.99 (1H, d, J = 6.04 Hz, H-1′), 5.19 (1H, apparent t, H-2′), 4.51 (1H, m, H-3′), 4.25-3.93 (3H, m, H-4′, 2H-5′), 3.10 (13.8H, q, J = 7.33 Hz, *CH*$_2$ TEA), 1.19 (19.6H, t, J = 7.33 Hz, *CH*$_3$ TEA); m/z (ESI) 423.9670 [M-H]$^-$, $C_{10}H_{12}BrN_5O_7P^-$ requires 423.9663; TLC: R_f 0.27 (^{t}BuOH/1M TEAB buffer 6:4).

8-(pyridin-3-yl) adenosine monophosphate (5a). The title compound was prepared according to the general synthetic method (i) from **4** (17.5mg, 1 equiv.) using Na_2PdCl_4 (2mol %), TPPTS (3 equiv. to Pd), 3-pyridinyl boronic acid (1.2 equiv.) and K_2CO_3 (3 equiv.) in water at 100 ºC. After 24h, the reaction was complete. The crude was purified by purification method 1 (0-10% MeOH against 0.05M TEAB buffer over 400mL, flow: 3mL/min, fraction size: 5mL) and 2 (0-25% 1M TEAB buffer against H_2O over 350mL, flow: 2ml/min, fraction size: 3mL). The product was isolated as a colorless oil (12.3mg, 2 equiv. TEA, 73% yield). ^{1}H NMR (400MHz, D_2O) δ: 8.82 (1H, br s, H-2 *pyridine*), 8.70 (1H, br s, H-6 *pyridine*), 8.22 (1H, s, H-2), 8.18 (1H, m, H-4 *pyridine*), 7.64 (1H, br s, H-5 *pyridine*), 5.77 (1H, d, H-1′), 5.22 (1H, apparent t, H-2′), 4.38 (1H, m, H-3′), 4.27-3.88 (3H, m, H-4′, 2H-5′), 3.14 (12.5H, q, J = 7.33 Hz, *CH*$_2$ TEA), 1.21 (18.6H, t, J = 7.33 Hz, *CH*$_3$ TEA); ^{31}P NMR (162MHz, D_2O) δ: 7.013; TLC: R_f 0.34 (^{t}BuOH/1M TEAB buffer 6:4).

8-(phenyl) adenosine monophosphate (5b). The title compound was prepared according to the general synthetic method (i) from **4** (83.3mg, 1 equiv.) using Na_2PdCl_4 (2.5mol %), TPPTS (2.5 equiv. to Pd), phenyl boronic acid (1.7 equiv.) and K_2CO_3 (1.5 equiv.) in water at 80 ºC. After 1h, the reaction was complete. The crude was purified by purification method 1 (0-60% MeOH against 0.05M TEAB buffer over 400mL, flow: 3mL/min, fraction size: 5mL) and 2 (0-60% 1M TEAB buffer against H_2O over 800mL, flow: 4ml/min, fraction size: 5mL). The product was isolated as colorless oil (75.1mg, 1.6 equiv. TEA, 82% yield). ^{1}H NMR (400MHz, D_2O) δ: 8.18 (1H, s, H-2), 7.68-7.49 (5H, m, *Ph*), 5.81 (1H, d, J = 6.23 Hz, H-1′), 5.21 (1H, apparent t, H-2′), 4.38 (1H, m, H-3′), 4.01 (3H, m, H-4′, 2H-5′), 2.97 (7.8H, q, J = 7.33 Hz, *CH*$_2$ TEA), 1.20 (16.4H, t, J = 7.33 Hz, *CH*$_3$ TEA); ^{31}P NMR (162MHz, D_2O) δ: 7.007; m/z (ESI) 422.0869 [M-H]$^-$, $C_{10}H_{12}BrN_5O_7P^-$ requires 422.0871; TLC: R_f 0.6 (^{i}PrOH/H_2O/NH_4OH 6:3:1).

8-(3-(*Boc*-aminomethyl)phenyl) adenosine monophosphate (5c). The title compound was prepared according to the general synthetic method (i) from **4** (74mg, 1 equiv.) using Na_2PdCl_4 (2.5mol %), TPPTS (2.5 equiv. to Pd), 3-(*N*-Boc-aminomethyl) phenyl boronic acid (1.2 equiv.) and K_2CO_3 (3 equiv.) in water at 80 ºC. After 1h, the reaction was complete. The crude was purified by purification method 1 (0-50% MeOH against 0.05M TEAB buffer over 400mL, flow: 3mL/min, fraction size: 5mL). The product was isolated as colorless oil (76.2mg, 1.3 equiv. TEA, 78% yield). ^{1}H NMR (400MHz, CD_3OD) δ: 8.18 (1H, s, H-2), 7.68-7.63 (2H, m, *Ph*), 7.55-7.46 (2H, m, *Ph*), 5.83 (1H, d, J = 5.83, H-1′), 5.51 (1H, apparent t, H-2′), 4.49 (1H, m, H-3′), 4.31 (2H, s, *CH*$_2$NH$_2$), 4.27-4.03 (3H, m, H-4′, 2H-5′), 3.01 (8H, q, *CH*$_2$ TEA), 1.42 (9H, s, *CH*$_3$ Boc), 1.19 (12.1H, t, *CH*$_3$ TEA); ^{31}P NMR (162MHz, D_2O) δ: 7.013; TLC: R_f 0.64 (^{t}BuOH/1M TEAB buffer 6:4).

8-(2,4-dimethoxy-5-pyrimidinyl) adenosine monophosphate (5d). The title compound was prepared according to the general synthetic method (i) from **4** (67.5mg, 1 equiv.) using Na_2PdCl_4 (2.5mol %), TPPTS (2.5 equiv. to Pd), 2,4-dimethoxy-5-pyrimidinyl boronic acid (1.2 equiv.) and K_2CO_3 (3 equiv.) in water at 80 ºC. After 1h, the reaction was complete. The crude was purified by purification method 1 (0-40% MeOH against 0.05M TEAB buffer over 400mL, flow: 3mL/min, fraction size: 5mL). The product was isolated as a colorless oil (74mg, 1.4 equiv. TEA, 75% yield). ^{1}H NMR (400MHz, D_2O) δ: 8.43 (1H, s, H-6 *pyrimidine*), 8.23 (1H, s, H-2), 5.56 (1H, d, J = 6.23, H-1′), 5.19 (1H, apparent t, H-2′), 4.44 (1H, m, H-3′), 4.14-3.82 (9H, m, H-4′, 2H-5′, 2 *OCH*$_3$), 2.83 (8.4H, q, J = 7.33 Hz, *CH*$_2$ TEA), 1.09 (12.3H, t, J = 7.33 Hz, *CH*$_3$ TEA); ^{31}P NMR (162MHz, D_2O) δ: 7.000; m/z (ESI) 484.0976 [M-H]$^-$, $C_{16}H_{19}N_7O_9P^-$ requires 484.0987; TLC: R_f 0.35 (^{t}BuOH/1M TEAB buffer 6:4).

8-(pyrrol-2-yl) adenosine monophosphate (5e). The title compound was prepared according to the general synthetic method (i) from **4** (17.5mg, 1 equiv.) using Na_2PdCl_4 (2mol %), TPPTS (3 equiv. to Pd), *N*-Boc-pyrrole-2-boronic acid (1.5 equiv.) and K_2CO_3 (3 equiv.) in water at 100 ºC. After 24h, the reaction was complete. The crude was purified by purification method 1 (0-12% MeOH against 0.05M TEAB buffer over 150mL, 12-40% over 200mL, flow: 3mL/min, fraction size: 5mL) and 2 (0-5% 1M TEAB buffer against H_2O over 50mL, 5-28% over 75mL, 28-40% over 20mL, isocratic 40% over 20mL, flow: 2ml/min, fraction size: 5mL). The product was isolated as colorless oil (9.8mg, 2.2 equiv. TEA, 57% yield). ^{1}H NMR (400MHz, CD_3OD) δ: 8.17 (1H, s, H-2), 7.16 (1H, dd, $J_{5,4}$ = 2.75 Hz, $J_{5,3}$ = 1.47 Hz, H-5 *pyrrole*), 6.85 (1H, dd, $J_{3,4}$ = 3.66 Hz, $J_{3,5}$ = 1.47 Hz, H-3 *pyrrole*), 6.32 (1H, dd, $J_{4,3}$ = 3.66 Hz, $J_{4,5}$ = 2.74 Hz, H-4 *pyrrole*), 6.17 (1H, d, J = 5.68 Hz, H-1′), 5.37 (1H, apparent t, H-2′), 4.55 (1H, m, H-3′), 4.34-4.03 (3H, m, H-4′, 2H-5′), 3.14 (13.8H, q, *CH*$_2$ TEA), 1.28 (19.6H, t, *CH*$_3$ TEA); ^{31}P NMR (162MHz, D_2O) δ: 7.032; m/z (ESI) 411.0812 [M-H]$^-$, $C_{14}H_{16}N_6O_7P^-$ requires 411.2871; TLC: R_f 0.65 (^{t}BuOH/1M TEAB buffer 6:4).

8-(pyridin-3-yl) NAD (3a). The title compound was prepared according to the general synthetic method (iii) from **6a** (14.3mg, 1 equiv.) and β-NMN (1.1 equiv.). The reaction was stirred

overnight. The crude was purified by purification method 1 (0-10% MeOH over 300mL, then 10-12% MeOH over 100mL, flow: 3mL/min, fraction size: 5mL). The product was isolated as a colorless oil (6.8mg, 1.18 equiv. TEA, 24% yield). ^{1}H NMR (400MHz, D_2O) δ: 9.29 (1H, s, H-2_N), 9.14 (1H, d, $J_{6,5}$ = 6.05, H-6_N), 9.01-8.65 (3H, m, H-4_N, H-2,6 *pyridine*), 8.34-8.13 (3H, m, H-2, H-5_N, H-4 *pyridine*), 7.70 (1H, br s, H-5 *pyridine*), 6.01 (1H, d, J = 5.13, H-1″), 5.80 (1H, d, J = 5.67, H-1′), 5.24 (1H, apparent t, H-2′), 4.51-4.11 (9H, m, H-3″, H-4″, 2H-5″, H-2″, H-3′, H-4′, 2H-5′), 3.19 (6.9H, q, *CH_2* TEA), 1.27 (10.6H, t, *CH_3* TEA); ^{13}C NMR (150.90MHz, D_2O) δ: 155.89, 153.53, 151.01, 150.53, 149.62, 146.60, 143.28, 140.56, 139.32, 134.53, 129.60, 119.50, 100.89, 89.71, 87.92, 84.08, 78.44, 71.38, 70.28, 66.41, 65.65, 47.52, 9.07; m/z (ESI) 739.1300 $[M-H]^-$, $C_{26}H_{29}N_8O_{14}P_2^-$ requires 739.1284; HPLC: retention time 7.07mins (95.8 %); TLC: R_f 0.62 (^{i}PrOH/H_2O/NH_4OH).

8-phenyl NAD (3b) [5]. The compound **3b** was prepared according to the general synthetic method (iii) from **6b** (1.2 equiv.) and β-NMN (3.8mg, 1equiv.). The reaction was stirred overnight. The crude was purified by purification method 1 (0% MeOH over 50mL, 0-40% MeOH over 350mL, flow: 3mL/min, fraction size: 5mL). The product was isolated as a colorless oil (4mg, 1.18 equiv. TEA, 41% yield). ^{1}H NMR (400MHz, D_2O) δ: 9.21 (1H, s, H-2_N), 9.06 (1H, d, $J_{6,5}$ = 6.41, H-6_N), 8.68 (1H, m, $J_{4,5}$ = 8.06, $J_{4.6}$ = 1.28, $J_{4,2}$ = 1.46, H-4_N), 8.18 (1H, s, H-2), 8.16-8.10 (1H, m, H-5_N), 7.65-7.50 (5H, m, *Ph*), 5.90 (1H, d, J = 5.13, H-1″), 5.79 (1H, d, J = 5.86, H-1′), 5.14 (1H, apparent t, H-2′), 4.43-4.06 (9H, m, H-3″, H-4″, 2H-5″, H-2″, H-3′, H-4′, 2H-5′), 3.14 (6.8H, q, *CH_2* TEA), 1.22 (10.6H, t, *CH_3* TEA); ^{13}C NMR (150.90MHz, D_2O) δ: 154.05, 153.70, 153.30, 150.91, 146.55, 143.18, 140.61, 132.04, 130.39, 130.25, 129.92, 129.63, 128.46, 119.14, 100.88, 89.82, 89.61, 87.85, 83.78, 78.41, 71.32, 71.23, 71.14, 70.38, 70.23, 66.61, 65.69, 59.73, 47.51, 9.07; ^{31}P NMR (162MHz, D_2O) δ: -11.7 (d, $J_{P,P}$ = 20.6Hz); -11.3 (d, $J_{P,P}$ = 20.6Hz); m/z (ESI) 738.1332 $[M-H]^-$, $C_{27}H_{30}N_7O_{14}P_2^-$ requires 738.1331; HPLC: retention time 10.34mins (79.4 %); TLC: R_f 0.43 (^{i}PrOH/H_2O/NH_4OH 6:3:1).

8-(3-(*Boc*-aminomethyl) phenyl) NAD (3c). The title compound was prepared according to the general synthetic method (iii) from **6c** (1.2 equiv.) and β-NMN (17.6mg, 1 equiv.). The reaction was stirred overnight. The crude was purified by purification method 1 (0-10% MeOH over 190mL, 10-30% over 50mL, isocratic 30% over 50mL, flow: 3mL/min, fraction size: 5mL). The product was isolated as a colorless oil (33.1mg, 1.7 equiv. TEA, 61% yield) and treated with Chelex to obtain the sodium salt. ^{1}H NMR (400MHz, D_2O) δ: 9.16 (1H, s, H-2_N), 8.99 (1H, br s, H-6_N), 8.60 (1H, m, H-4_N), 8.07 (2H, m, H-2, H-5_N), 7.54-7.26 (4H, m, *Ph*), 5.85 (1H, br s, H-1″), 5.71 (1H, br s, H-1′), 5.16 (1H, br s, H-2′), 4.49-3.77 (11H, m, H-3″, H-4″, 2H-5″, H-2″, H-3′, H-4′, 2H-5′, *CH_2*NH_2), 1.33 (9H, s, *CH_3* Boc); ^{13}C NMR (150.90MHz, D_2O) δ: 158.95, 155.55, 153.21, 152.45, 150.65, 148.28, 146.33, 142.95, 140.67, 140.41, 137.07, 134.10, 130.20-128.23, 124.83, 118.92, 101.81, 100.66, 96.99, 89.71, 87.67, 83.67, 81.92, 78.22, 75.81, 71.34-70.15, 66.34, 65.39, 44.10, 28.36; ^{31}P NMR (162MHz, D_2O) δ: -11.3 (d, $J_{P,P}$ = 20.6Hz); -11.1 (d, $J_{P,P}$ = 20.6Hz); m/z (ESI) 867.2120 $[M-H]^-$, $C_{33}H_{41}N_8O_{16}P_2^-$ requires 867.2121; HPLC: retention time 17.96mins (99.5 %); TLC: R_f 0.75 (^{i}PrOH/H_2O/ NH_4OH 6:3:1).

8-(2,4-dimethoxy-5-pyrimidinyl) NAD (3d). The title compound was prepared according to the general synthetic method (iii) from **6d** (1.5 equiv.) and β-NMN (11.2mg, 1 equiv.). The reaction was stirred overnight. The crude was purified by purification method 1 (0-22% MeOH over 300mL, 22-40% over 50mL, isocratic 40% over 50mL, flow: 3mL/min, fraction size: 5mL). The product was isolated as a colorless oil (13.9mg, 1.7eq TEA, 43% yield) and treated with Chelex to obtain the sodium salt. ^{1}H NMR (400MHz, D_2O) δ: 9.25 (1H, s, H-2_N), 9.08 (1H, m, H-6_N), 8.68 (1H, m, H-4_N), 8.33 (1H, m, H-6 *pyrimidine*), 8.10 (2H, br s, H-2, H-5_N), 6.01 (1H, d, H-1″), 5.49 (1H, d, H-1′), 5.08 (1H, apparent t, H-2′), 4.53-3.85 (15H, m, H-3″, H-4″, 2H-5″, H-2″, H-3′, H-4′, 2H-5′, 2 *OCH_3*); ^{13}C NMR (150.90MHz, D_2O) δ: 169.85, 167.00, 161.02, 155.64, 153.60, 152.48, 150.47, 148.31, 146.40, 143.03, 137.11, 134.25, 129.37, 124.87, 119.49, 104.96, 101.81, 100.69, 97.02, 90.06, 87.71, 83.85, 81.78, 78.28, 75.82, 71.78-70.43, 66.90-65.50, 59.66, 56.29,

55.60; ^{31}P NMR (162MHz, D_2O) δ: -11.3 (d, $J_{P,P}$ = 20.6Hz); -11.1 (d, $J_{P,P}$ = 20.6Hz); m/z (ESI) 800.1447 $[M-H]^-$, $C_{27}H_{32}N_9O_{16}P_2^-$ requires 800.1448; HPLC: retention time 11.41mins (96.5 %); TLC: R_f 0.69 (^{i}PrOH/H_2O/ NH_4OH 6:3:1).

8-(pyrrol-2-yl) NAD (3e). The title compound was prepared according to the general synthetic method (iii) from **6e** (1.5 equiv.) and β-NMN (33.2mg, 1 equiv.). The reaction was stirred overnight. The crude was purified by purification method 1 (0-10% MeOH over 400mL, flow: 3mL/min, fraction size: 5mL) and 2 (0-80% 1M TEAB buffer against H_2O over 400mL, flow: 3ml/min, fraction size: 5mL). The product was isolated as a colorless oil (24.5mg, 1.01 equiv. TEA, 30% yield). ^{1}H NMR (400MHz, D_2O) δ: 9.09 (1H, s, H-2_N), 8.92 (1H, d, $J_{5,6}$ = 6.16Hz, H-6_N), 8.60 (1H, d, $J_{4,5}$ = 8.1Hz, H-4_N), 8.09 (1H, s, H-2), 8.05 (1H, dd, J = 6.48 and 7.82Hz, H-5_N), 7.05 (1H, dd, $J_{5,4}$ = 2.37Hz, $J_{5,3}$ = 1.21Hz, H-5 *pyrrole*), 6.57 (1H, dd, $J_{3,4}$ = 3.64Hz, $J_{3,5}$ = 1.07Hz, H-3 *pyrrole*), 6.24 (1H, dd, J = 3.43 and 2.86Hz, H-4 *pyrrole*), 5.91 (1H, d, J = 6.06Hz, H-1″), 5.73 (1H, d, J = 4.82Hz, H-1′), 5.33 (1H, apparent t, H-2′), 4.54 (1H, dd, J = 4.56 and 6.09Hz, H-2″), 4.41-3.98 (8H, m, H-3″, H-4″, 2H-5″, H-3′, H-4′, 2H-5′), 3.15 (6.08H, q, CH_2 TEA), 1.24 (9.14H, t, CH_3 TEA); ^{13}C NMR (150.90MHz, D_2O) δ: 154.89, 151.98, 150.90, 147.10, 146.40, 142.91, 140.48, 134.14, 129.64, 124.27, 119.43, 113.82, 110.00, 100.81, 89.46, 87.79, 87.73, 83.81, 83.76, 78.41, 71.24, 70.89, 70.02, 66.47, 65.61, 59.60, 47.52, 9.07; ^{31}P NMR (162MHz, D_2O) δ: -11.9 (d, $J_{P,P}$ = 20.9Hz); -11.4 (d, $J_{P,P}$ = 21.0Hz); m/z (ESI) 727.1276 $[M-H]^-$, $C_{25}H_{29}N_8O_{14}P_2^-$ requires 727.1284; HPLC: retention time 7.68mins (99.8 %).

References for this section:

[1] (a) E. C. Western, J. R. Daft, E. M. Johnson, P. M. Gannett, K. H. Shaughnessy, *J. Org. Chem.* **2003**, *68*, 6767; (b) P. Capek, R. Pohl, M. Hocek, *Org. Biomol. Chem.* **2006**, *4*, 2278; (c) A. Collier, G. K. Wagner, *Chem. Commun.* **2008**, 178; (d) T. Pesnot, G. K. Wagner, *Org. Biomol. Chem.* **2008**, *6*, 2884.

[2] T. Mukaiyama, M. Hashimoto, *Bull. Chem. Soc. Jpn.* **1971**, *44*, 2284.

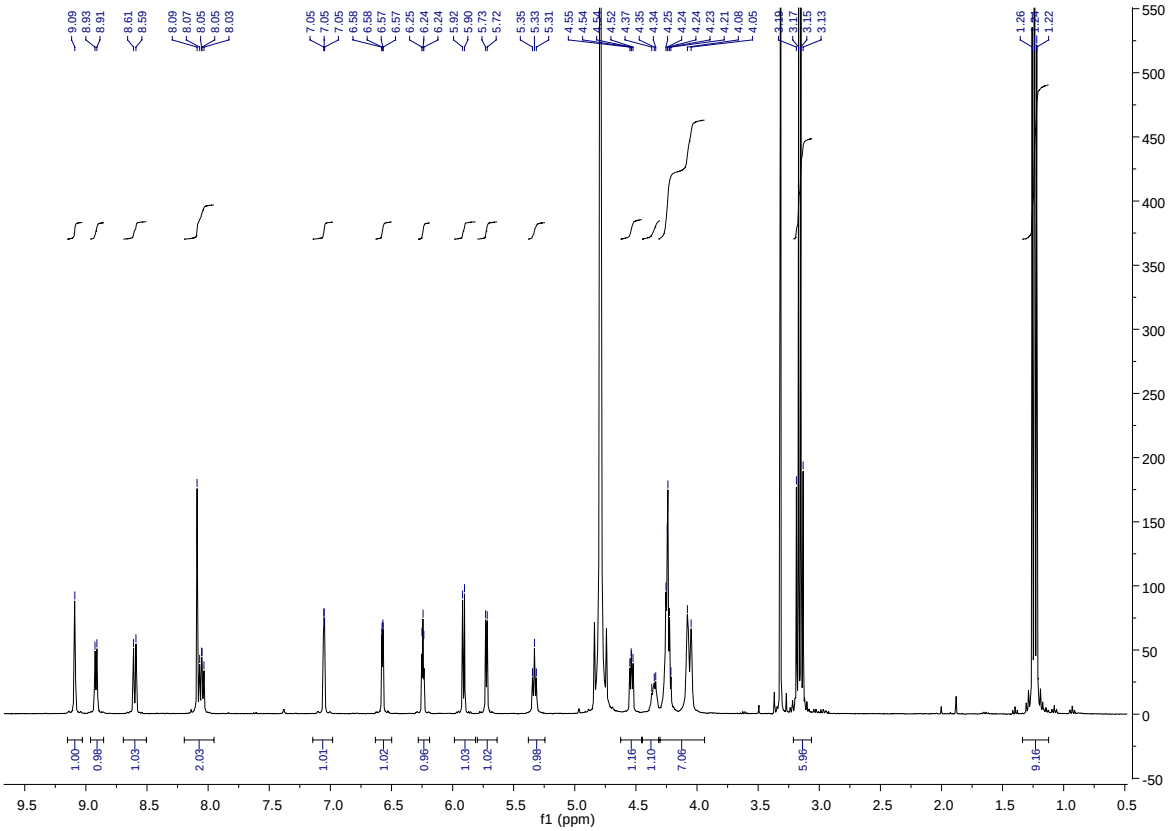
[3] J. Lee, H. Churchil, W.-B. Choi, J. E. Lynch, F. E. Roberts, R. P. Volante, P. J. Reider, *Chem. Commun.* **1999**, 729.

[4] M. Ikehara, S. Uesugi, M. Kaneko, *Chem. Commun.* **1967**, 17.

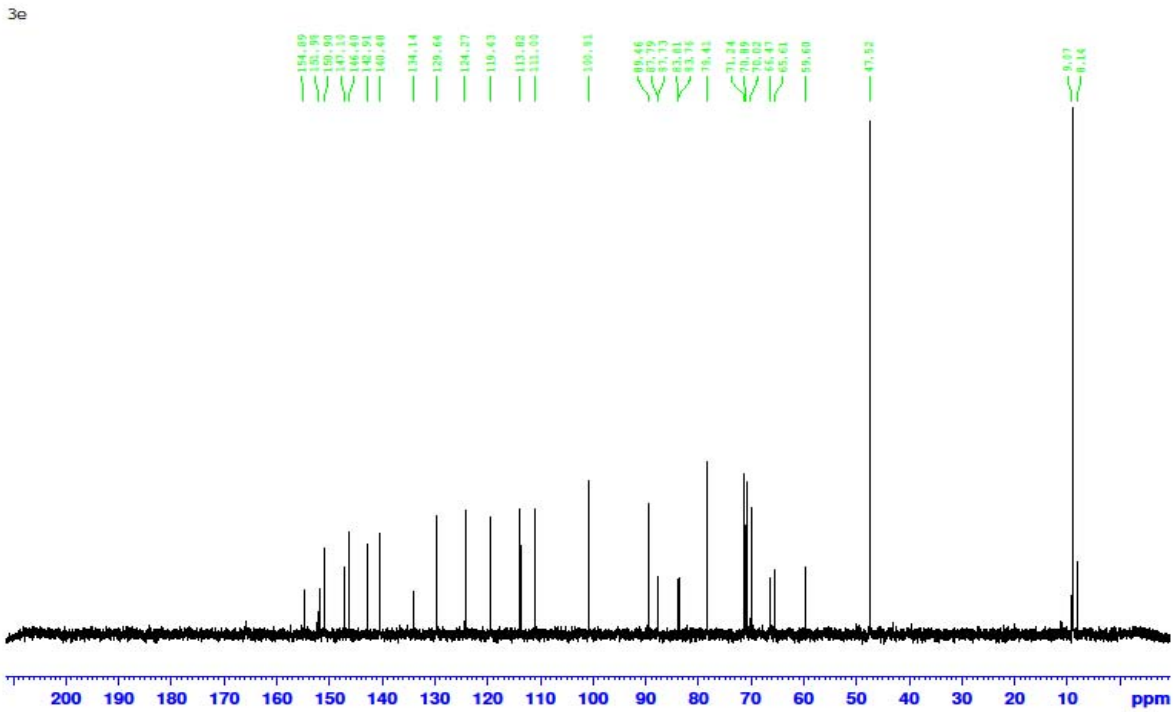
[5] T. Pesnot, J. Kempter, J. Schemies, G. Pergolizzi, U. Uciechowska, T. Rumpf, W. Sippl, M. Jung, G. K. Wagner, *J. Med. Chem.* **2011**, *54*, 3492.

(iii) NMR SPECTRA OF 8-(PYRROL-2-YL) NAD **3e**

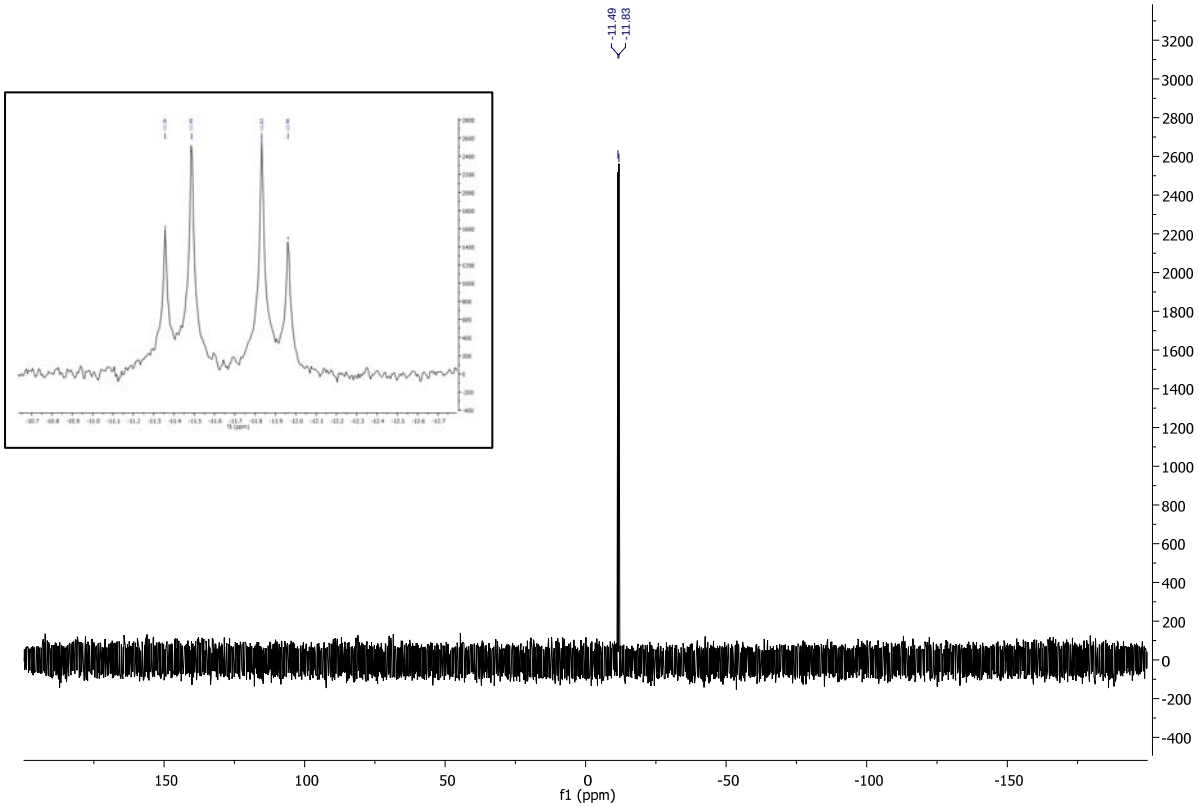
^{1}H NMR:



^{13}C NMR:



^{31}P NMR:


-11.49
-11.83
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
-400
150
100
50
0
-50
-100
-150
f1 (ppm)

(iv) PROTOCOLS FOR ENZYME ASSAYS

Protocols for HPLC assays of enzyme activities

Nucleotide pyrophosphatase (NPP). Separate stock solutions of **3e** and NPP from *Crotalus adamanteus* venom (8.39U/mL) were prepared in Tris/HCl buffer (50mM, pH 8) containing $MgCl_2$ (10mM). The reaction was started by addition of an appropriate volume of **3e** stock (24.7μM) to the enzyme solution (0.01U/mL). The reaction was incubated at 30ºC for 90mins. Samples were drawn at different time points and stored immediately on dry-ice, for 20mins, to stop the enzymatic reaction. Samples were diluted as appropriate and analysed by HPLC (Analytical method as described; retention times: **3e** 6.5mins, β-NMN 1.8mins, **5e** 7.7mins). The estimated rate constant *k* for the formation of **5e** was obtained by fitting the peak areas to a 1st order rate equation (*k* 0.2457 min^{-1}, $t_{1/2}$ 2.82mins).

ADP-ribosyl cyclase (ADPRC). Separate stock solutions of **3e** and ADPRC from *Aplysia californica* (50U/mL) were prepared in HEPES buffer (50mM, pH 7.4). The reaction was started by addition of an appropriate volume of **3e** stock (12.4μM) to the enzyme solution (0.05U/mL). The reaction was incubated at 26ºC for 1h. Samples were drawn at different time points and stored immediately on dry-ice, for 20mins, to stop the enzymatic reaction. Samples were diluted as appropriate and analysed by HPLC (Analytical method as described; retention times: **3e** 6.6mins, nicotinamide 3.8mins, cADPR-**3e** 8.7mins). The estimated rate constants k_{nic} for the formation of nicotinamide, and $k_{cADPR\text{-}3e}$ for the formation of cADPR-**3e**, were obtained by fitting the peak areas to a 1st order rate equation (k_{nic} 0.3525min^{-1}, $t_{1/2}$ 1.97mins; $k_{cADPR\text{-}3e}$ 0.1700 min^{-1}, $t_{1/2}$ 4.08mins).

NAD-glycohydrolase (NGH). A stock solution of **3e** was prepared in Tris/HCl buffer (50mM, pH 8). NGH from porcine brain (0.01U/mg) was accurately weighed and suspended in Tris/HCl buffer (50mM, pH 8) and incubated for a few minutes at 37 ºC. The reaction was started by addition of an appropriate volume of **3e** stock (24.74μM) to the enzyme suspension (0.009U/mL). Samples were drawn at different time points and stored immediately on dry-ice, for 20mins, to stop the enzymatic reaction. Samples were centrifuged, diluted as appropriate and analysed by HPLC (Analytical method as described; retention times: **3e** 7.3mins, nicotinamide 3.8mins, ADPR-**3e** 8.0mins). The estimated rate constants k_{nic} for the formation of nicotinamide, and $k_{ADPR\text{-}3e}$ for the formation of ADPR-**3e**, were obtained by fitting the peak areas to a 1st order rate equation (k_{nic} 0.1750min^{-1}, $t_{1/2}$ 3.96mins; $k_{ADPR\text{-}3e}$ 0.1696min^{-1}, $t_{1/2}$ = 4.09mins). In a control experiment, **3e** was incubated in Tris/HCl buffer (50mM, pH 8) at 37 ºC over 1h without degradation.

Protocol for fluorescence assays of enzyme activities

Nucleotide pyrophosphatase (NPP). Separate stock solutions of **3e**, **5e** and NPP from *Crotalus adamanteus* venom (8.39U/mL) were prepared in Tris/HCl buffer (50mM, pH 8) containing $MgCl_2$ (10mM), unless stated otherwise. In the kinetic experiments, the requisite volume of substrate **3e** or product **5e** was added to individual wells of a 96-well plate, and Tris/HCl buffer (50mM, pH 8) containing $MgCl_2$ (10mM) was added as required to give a final volume of 100μL. In each experiment, two calibration wells were included for the determination of sample concentration from fluorescence intensity: one well contained only Tris/HCl buffer (50mM, pH 8) and $MgCl_2$ (10mM) and gave the 'zero' readout, and the second contained **5e** (3μM) in Tris/HCl buffer (50mM, pH 8) and $MgCl_2$ (10mM). Samples and fluorimeter readings were allowed to stabilize by placing the plate in the multiplate reader at 30ºC and taking readings (excitation 300 ± 5nm, emission 410 ± 5nm) until a stable signal was obtained (typically ca 60mins). The data collection program was re-started, and after 7mins the enzyme reaction was initiated by addition of enzyme stock solution (20μL, 0.007U/mL per well). Fluorescence

emission was recorded for 60mins. All samples, including controls, were recorded in duplicate, and the average of the two readings processed. For the determination of K_m, enzyme (0.007U/mL) and **3e** (0.2 µM-6.5µM) in Tris/HCl buffer (50mM, pH 8) containing $MgCl_2$ (10mM) were used (total volume per well: 120µL, all concentrations are final concentrations).

ADP-ribosyl cyclase (ADPRC). Separate stock solutions of **3e** and ADPRC from *Aplysia californica* were prepared in HEPES buffer (50mM, pH 7.4), unless stated otherwise. In the kinetic experiments, the requisite volume of substrate **3e** was added to individual wells of a 96-well plate, and HEPES buffer (50mM, pH 7.4) was added as required to give a final volume of 198µL. In each experiment, two calibration wells were included for the determination of sample concentration from fluorescence intensity: one well contained only HEPES buffer (50mM, pH 7.4) and gave the 'zero' readout, and the second contained **5e** (34µM) in HEPES buffer (50mM, pH 7.4). Samples and fluorimeter readings were allowed to stabilize by placing the plate in the multiplate reader at 25ºC and taking readings (excitation 300 ± 5nm, emission 410 ± 5nm) until a stable signal was obtained (typically ca 40mins). The data collection program was re-started, and after 17mins the enzyme reaction was initiated by addition of enzyme stock solution (2µL, 0.075U/mL per well). Fluorescence emission was recorded for 80mins. All samples, including controls, were recorded in duplicate, and the average of the two readings processed. For the determination of K_m, enzyme (0.075U/mL) and **3e** (2.1 µM-33.9µM) in HEPES buffer (50mM, pH 7.4) were used (total volume per well: 200µL, all concentrations are final concentrations).

NAD-glycohydrolase (NGH). A stock solution of **3e** was prepared in Tris/HCl buffer (50mM, pH 8), unless stated otherwise. In the kinetic experiments, Tris/HCl buffer (50mM, pH 8, 150µL) was added to individual wells of a 96-well plate. In each experiment, two calibration wells were included for the determination of sample concentration from fluorescence intensity: one well contained only Tris/HCl buffer (50mM, pH 8) and gave the 'zero' readout, the second contained **5e** (5.25µM) in Tris/HCl buffer (50mM, pH 8). NGH from porcine brain (0.01U/mg) was accurately weighed and suspended in Tris/HCl buffer (50mM, pH 8) and incubated for several minutes at 37ºC. The reaction was started by addition of an appropriate volume of **3e** (5.25µM) to the enzyme suspension (0.0075U/mL). Samples (70µL) were drawn at different time points and 50µL added to the wells. Fluorescence emission was recorded at 37ºC (excitation 300 ± 5nm, emission 410 ± 5nm). All samples, including controls, were recorded in duplicate, and the average of the two readings processed.

(v) ADDITIONAL TABLES, FIGURES AND SCHEMES

Table S1 Fluorescence spectroscopic properties of 8-substituted NAD (**3a-e**) and AMP (**5a-e**) derivatives, etheno-NAD (**2**) and etheno-AMP in water.

Cmpd	R	λ_{ex}/nm	λ_{em}/nm	Stokes shift[a]/cm^{-1}
ε-NAD (**2**)[b]	*not applicable*	306	410	8289
ε-AMP[b]	*not applicable*	306	400	7680
3a	3-pyridinyl	273	401	11692
5a	3-pyridinyl	277	401	11163
3b	phenyl	272	381	10518
5b	phenyl	274	381	10250
3c	3-(*Boc*-aminomethyl)phenyl	274	383	10387
5c	3-(*Boc*-aminomethyl)phenyl	274	383	10387
3d	2,4-*DMT*-pyrimidinyl	268	412	13042
5d	2,4-*DMT*-pyrimidinyl	270	410	12647
3e	2-pyrrolyl	309	374	5624
5e	2-pyrrolyl	306	374	5942

[a]Stokes shift = $(1/\lambda_{excitation} - 1/\lambda_{emission})$; [b]from *Crit. Rev. Biochem. Mol. Biol.* **1984**, *15*, 125.

Table S2 Enzymological properties for NAD (**1**), ε-NAD (**2**) and the related dinucleotide ε-PdAD with different nucleotide pyrophosphatases.

Organism	NAD (**1**)		ε-NAD (**2**) or ε-PdAD	
	K_m (mM)	v_{max} (μmol·min^{-1}·mg^{-1})	K_m (mM)	v_{max} (μmol·min^{-1}·mg^{-1})
Haemophilus parasuis[a]	0.0041	13.5	0.144[e]	2.5[e]
Haemophilus influenzae[b]	0.0054	2.7	0.632[e]	3.6[e]
Potato[c]	0.05	n.a.[g]	0.013[e]	n.a.
Mouse splenocytes[d]	n.a.	n.a.	0.29-0.33[f]	n.a.

[a]D. J. Wise, C. D. Anderson, B. M. Anderson, *Vet. Microbiol.* **1997**, *58*, 261; [b]D. W. Kahn, B. M. Anderson, *J. Biol. Chem.* **1986**, *261*, 6016; [c]J. Wierzchowski, H. Sierakowska, D. Shugar, *BBA - Protein Struct. M.* **1985**, *828*, 109; [d]C. D. Muller, C. Tarnus, S. Schuber, *Biochem. J.* **1984**, *223*, 715; [e]ε-NAD; [f]ε-PdAD; [g]not available.

Table S3 Enzymological properties for NAD (**1**), **ε**-NAD (**2**), **3e** and other dinucleotide substrates of ADP-ribosyl cyclase.[a]

	NAD (**1**)	**3e**	**ε**-NAD (**2**)	NGD	NHD	NXD
K_m (μM)	39[b], 135[c]	74	6.2[b]	1.7[b], 15.5[c]	0.5[b], 8.8[c]	6.2[b]
V_{max} (μmol·min^{-1}·mg^{-1})	500[b]	21.8	94.4[b]	36.3[b]	4.6[b]	50[b]

[a]Abbreviations: NGD nicotinamide guanine dinucleotide; NHD nicotinamide hypoxanthine dinucleotide; NXD nicotinamide xanthine dinucleotide; [b]R. M. Graeff, T. F. Walseth, H. K. Hill, H. C. Lee, *Biochemistry* **1996**, *35*, 379; [c]M. E. Migaud, R. L. Pederick, V. C. Bailey, B. V. L. Potter, *Biochemistry* **1999**, *38*, 9105.

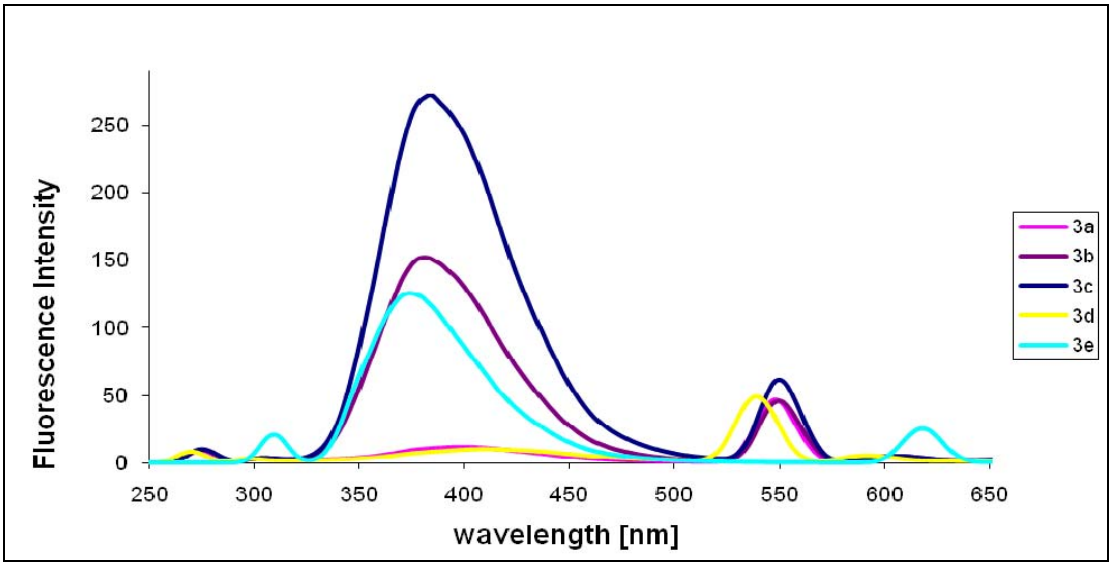


Fig. S1 Fluorescence emission spectra for NAD derivatives **3a-e** (0.8µM in water; excitation at λ_{max}, see Table S1)

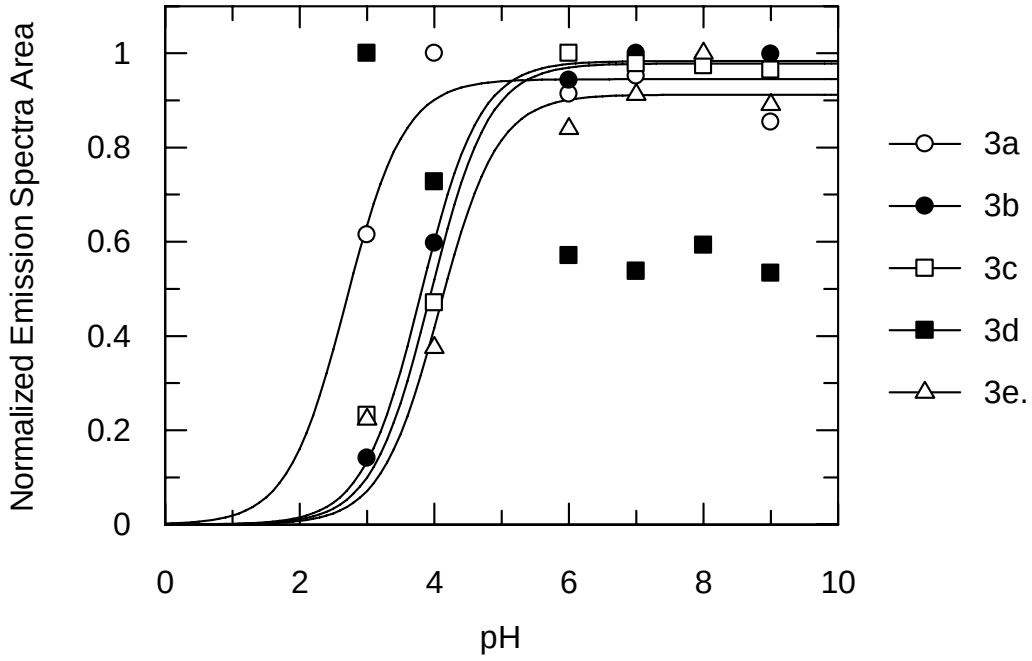


Fig. S2 Fluorescence intensity at different pH values for NAD derivatives **3a-e**.

Conditions: A sodium acetate buffer (50mM) with addition of acetic acid was used for pH 3 and 4. A potassium dihydrogen phosphate buffer (50mM) with addition of NaOH was used for pH 6. A Tris buffer (50mM) with addition of HCl was used for pH 7, 8 and 9. Measurements were performed at r.t. The compound concentrations were respectively: 3.6µM for **3a**, 1.01µM for **3b**, 3.9µM for **3c**, 3.5µM for **3d**, 0.81µM for **3e**. Emission spectra areas were normalized to the highest value and the values were fitted to the pK_a single curve equation using GraFit5 (except for **3d**). The estimated pK_a values were respectively: 2.69 for **3a**, 3.81 for **3b**, 3.95 for **3c**, 4.07 for **3e**.

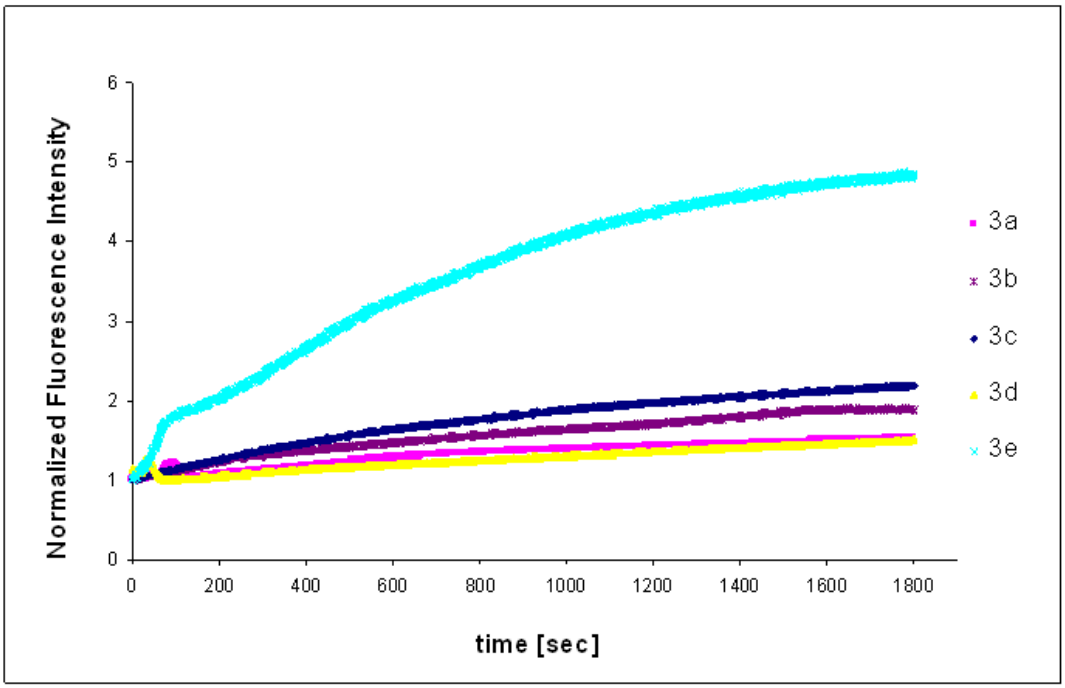


Fig. S3 Fluorescence time-course of basic hydrolysis for NAD derivatives **3a-e** (0.8µM in 0.1N NaOH, room temperature, λ_{ex} and λ_{em} for each compound as shown in Table S1)

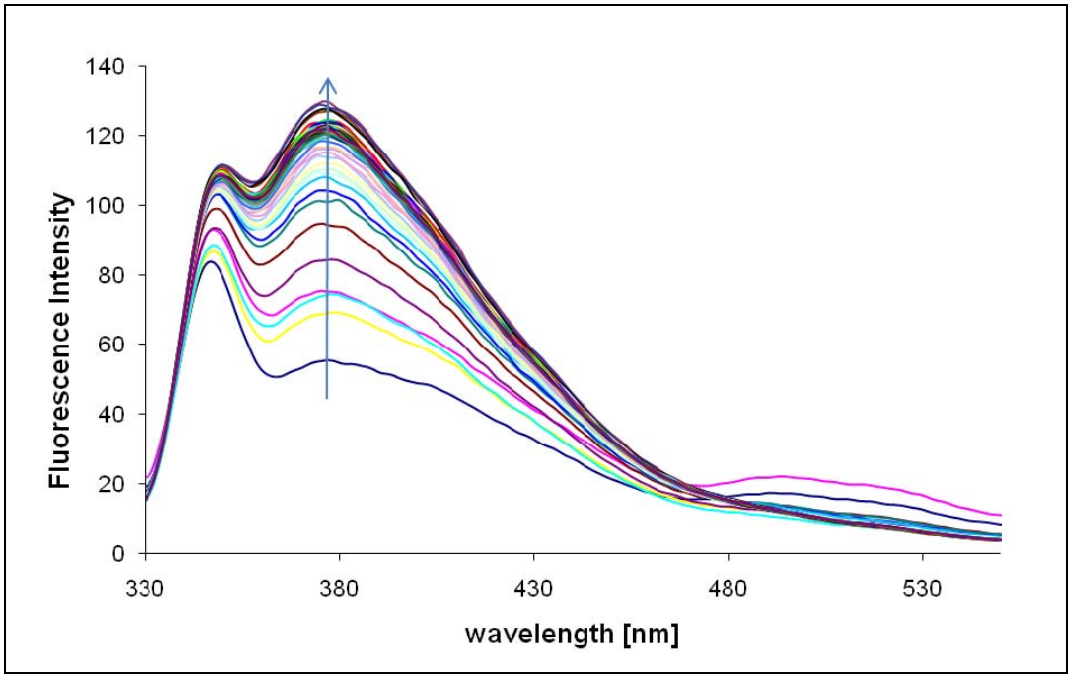


Fig. S4 Fluorescence time-course of basic hydrolysis for **3e** (0.8µM, in 0.1N NaOH, room temperature), monitoring the whole emission spectra at λ_{ex} 309nm. The arrow indicates the increase in fluorescence at 374nm over time.

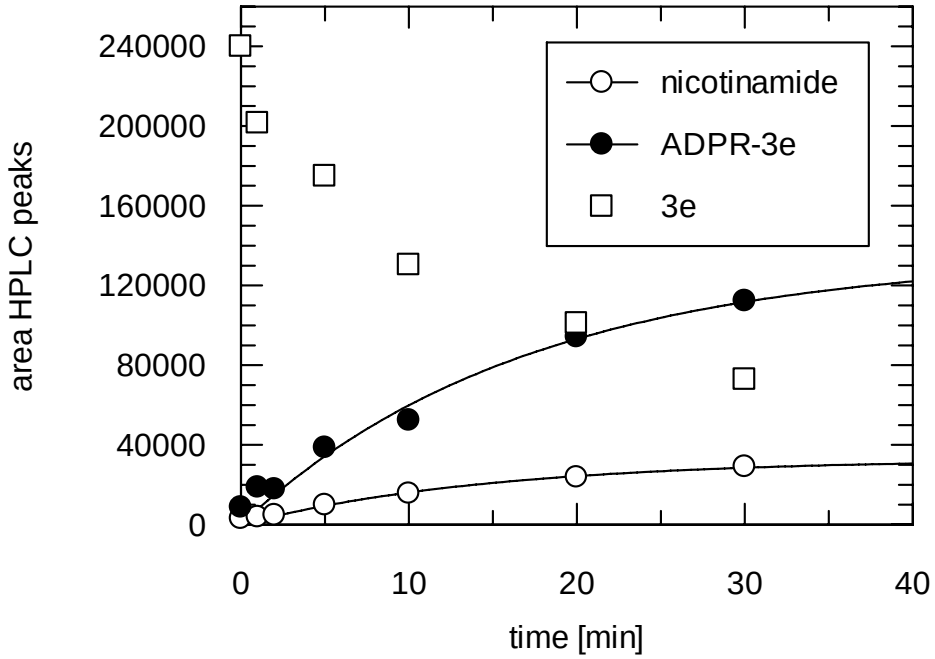


Fig. S5 HPLC control experiment for the basic hydrolysis of **3e** (49.5µM in 0.1N NaOH, room temperature). Each sample was quenched with an equivalent volume of 0.1N HCl prior to injection into the HPLC. Peak areas were fitted to a 1st order rate equation using GraFit5. The estimated rate constant for nicotinamide formation, k_{nic}, from fitting the peak areas to a 1st order rate equation is 0.0672min^{-1}, and the $t_{1/2}$ = 10.31mins, in agreement within error of each other with the estimated rate constant for formation of ADPR-**3e** k_{ADPR3e}, 0.0586min^{-1}, and the $t_{1/2}$ = 11.83mins.

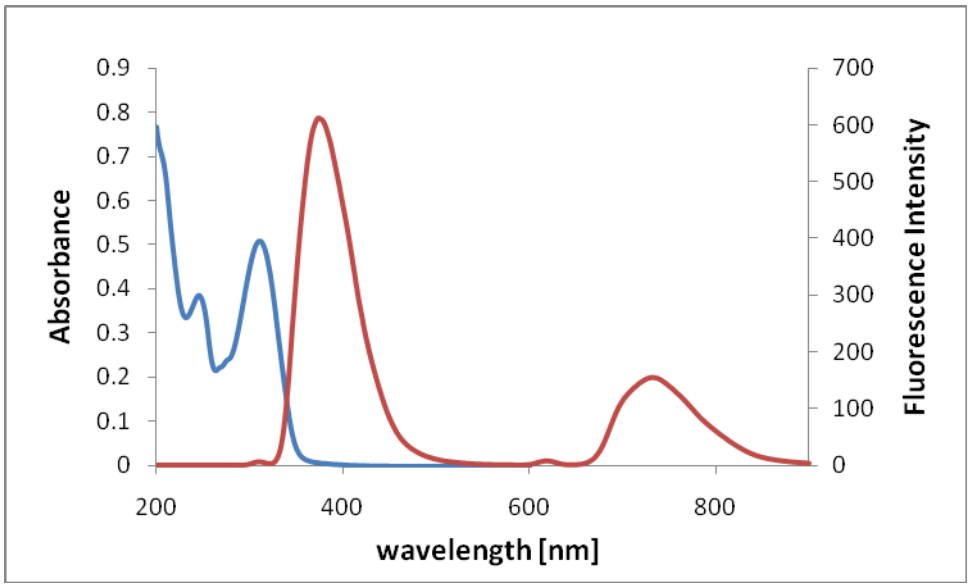


Fig. S6 Absorbance (blue) and emission (red) spectra of **3e** (4µM in water, λ_{ex} 309 nm). For comparison: excitation and emission maxima for tryptophan, the most common fluorophore in proteins, are 280nm and 350 nm respectively.

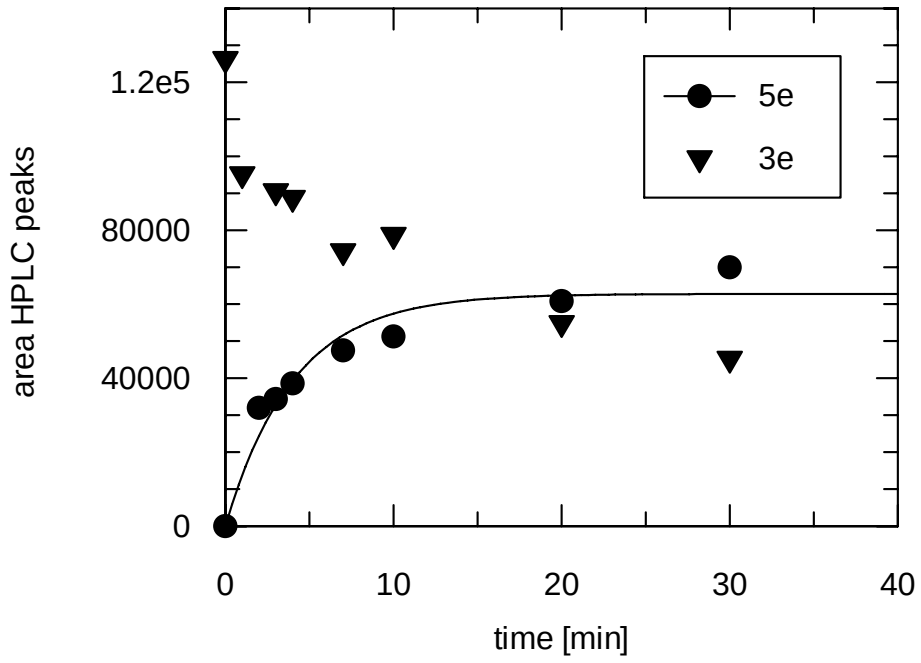


Fig. S7 HPLC assay of nucleotide pyrophosphatase (NPP) and **3e**. *Conditions*: enzyme (0.01U/mL), **3e** (24.7μM), $MgCl_2$ (10mM), Tris/HCl buffer (50mM, pH 8), 30ºC. The consumption of **3e** and the formation of **5e** were followed. Peak areas were fitted to a 1st order rate equation with GraFit5.

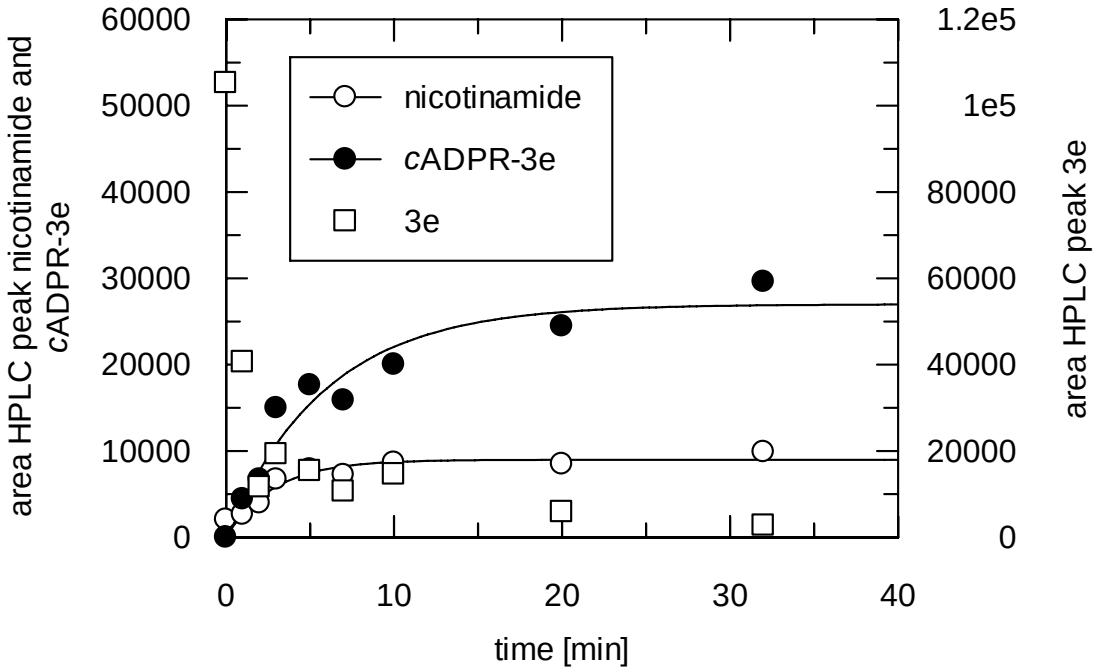


Fig. S8 HPLC assay of ADP-ribosyl cyclase (ADPRC) and **3e**. *Conditions*: enzyme (0.05U/mL), **3e** (12.4μM), HEPES buffer (50mM, pH 7.4). The consumption of **3e** and the formation of, respectively, nicotinamide and 8-pyrrolyl cADPR (cADPR-**3e**) were followed. Peak areas were fitted to a 1st order rate equation with GraFit5.

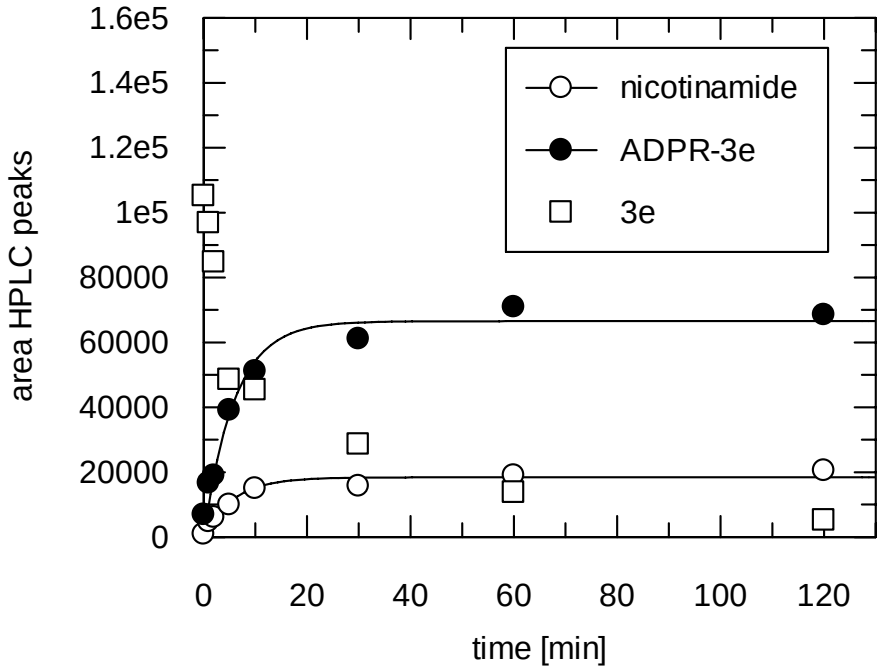


Fig. S9 HPLC assay of NAD-glycohydrolase (NGH) and **3e**. *Conditions*: enzyme (0.009U/mL), **3e** (24.74µM), Tris/HCl buffer (50mM, pH 8), 37 ºC. The consumption of **3e** and the formation of 8-pyrrolyl ADPR (ADPR-**3e**) were followed. Peak areas were fitted to a 1st order rate equation with GraFit5.

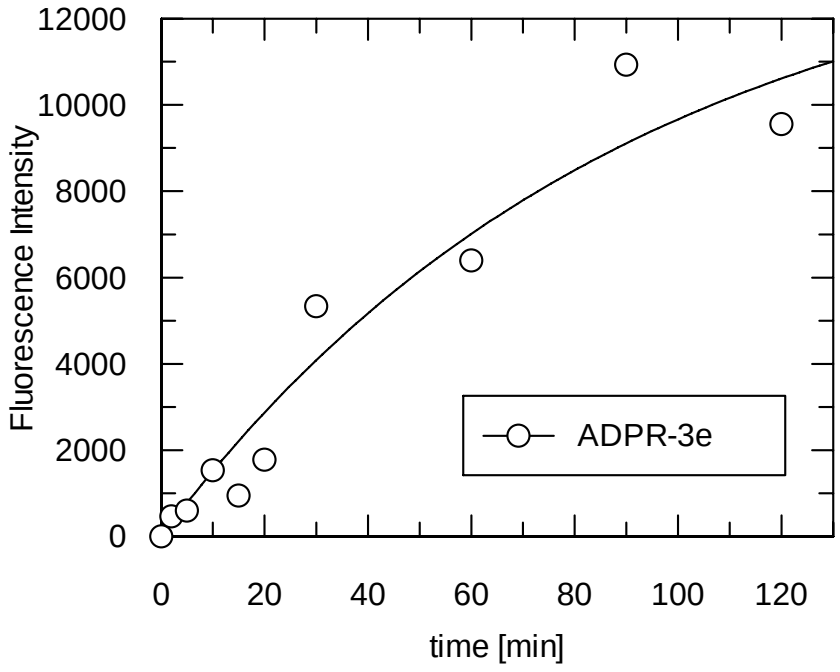


Fig. S10 Fluorimetric assay of NAD-glycohydrolase (NGH) and **3e**. *Conditions*: enzyme (0.0075U/mL), **3e** (5.25µM), Tris/HCl buffer (50mM, pH 8), 37 ºC, λ_{ex} 300nm, λ_{em} 410nm, gain 15%. Fluorescence intensity values were fitted to a 1st order rate equation with GraFit5. The estimated rate constant for the formation of ADPR-**3e**, $k_{ADPR\text{-}3e}$, is 0.0111min^{-1}, and the $t_{1/2}$ 62.45mins.

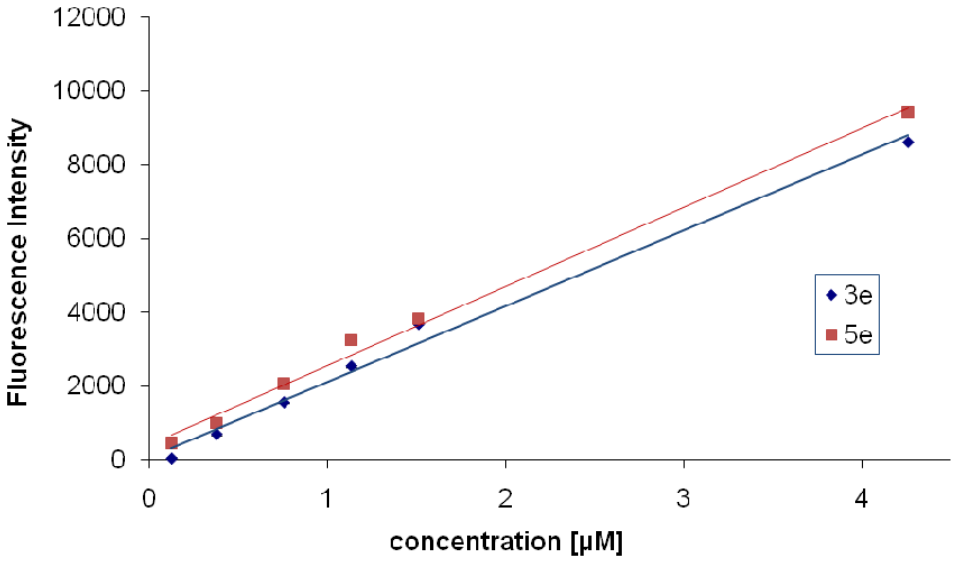


Fig. S11 Fluorescence calibration curves for **3e** and **5e.** *Conditions*: $MgCl_2$ (10mM), Tris/HCl buffer (50mM, pH 8), 30 ºC, λ_{ex} 300nm, λ_{em} 410nm, gain 15%. Fluorescence intensity values were fitted to a linear equation with Excel. The estimated linear equations are, respectively: y = 2149.4x + 418.93 (r^2 = 0.994) for **5e**, and y = 2052.9x + 62.892 (r^2 = 0.991) for **3e**.

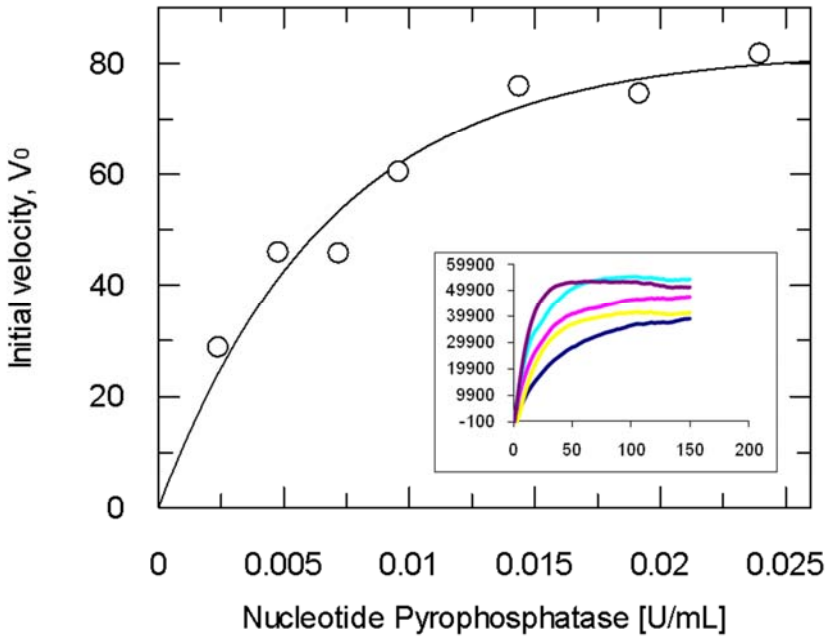


Fig. S12 Fluorimetric assay of nucleotide pyrophosphatase (NPP) and **3e**: **initial velocity v_0 as function of NPP concentration**. *Conditions*: enzyme (0.0024-0.024U/mL), **3e** (0.09µM), $MgCl_2$ (10mM), Tris/HCl buffer (50mM, pH 8), 30ºC, λ_{ex} 300nm, λ_{em} 410nm. Initial velocity values were calculated from the reaction time courses (insert) and based on the respective fluorescence signal at 10% conversion. Initial velocity values were fitted to a polynomial equation with GraFit5.

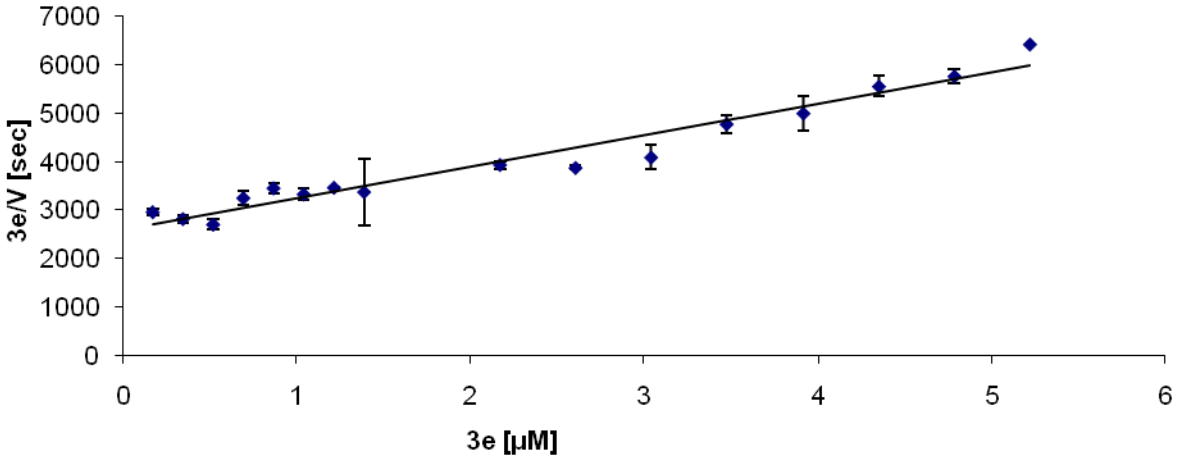


Fig. S13 Fluorimetric assay of nucleotide pyrophosphatase (NPP) and **3e**: **Hanes-Woolf plot**. *Conditions*: enzyme (0.007U/mL), **3e** (0.2-6.5µM), $MgCl_2$ (10mM), Tris/HCl buffer (50mM, pH 8), 30 ºC, λ_{ex} 300nm, λ_{em} 410nm, gain 15%. Initial velocity values were calculated from the time courses measuring the fluorescence signals generated when 10% product is formed. Initial velocity values were then fitted to the Hanes-Woolf plot with Excel. The estimated linear equation is $y = 651.08x + 2591.9$ ($r^2 = 0.9519$).

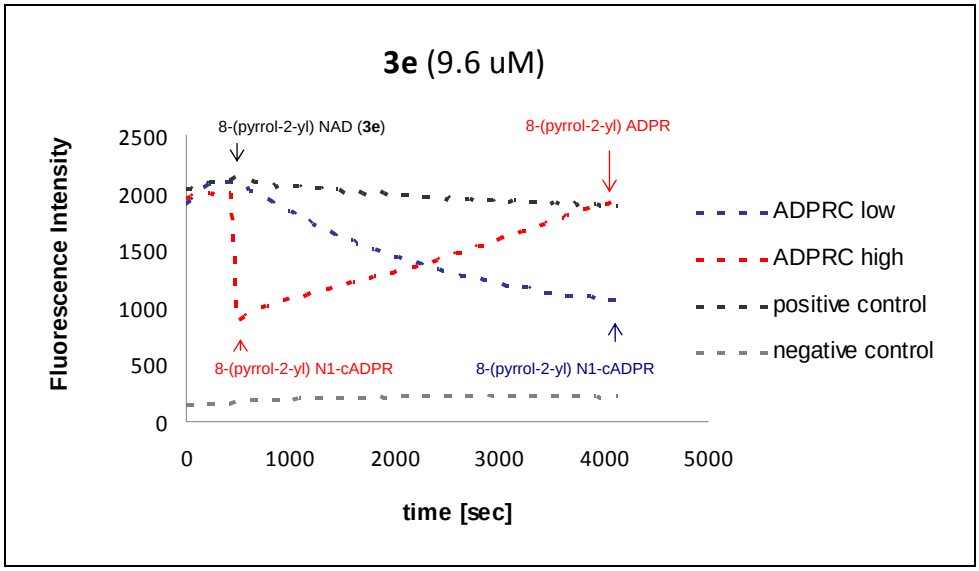


Fig. S14 Fluorimetric assay of ADP-ribosyl cyclase (ADPRC) and **3e**. *Conditions*: **3e** (9.6µM), ADPRC (blue line: 0.025U/mL, red line: 1.75U/mL), HEPES buffer (50mM, pH 7.4), 25°C, λ_{ex} 300nm, λ_{em} 410nm, gain 15%. Enzyme addition at 780sec. Positive control: **3e** only; negative control: buffer.

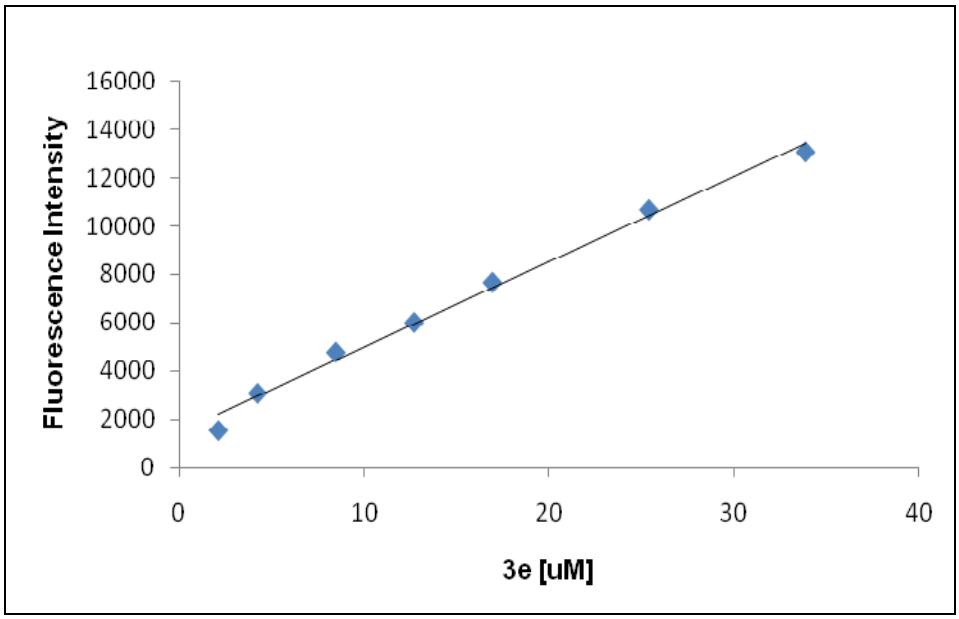


Fig. S15 Fluorescence calibration curve for **3e** in HEPES buffer (50mM, pH 7.4). *Other conditions*: 26 ºC, λ_{ex} 300nm, λ_{em} 410nm, gain 15%. Fluorescence intensity values were fitted to a linear equation with Excel. The estimated linear equation is y = 354.05x + 1437.3 (r^2 = 0.992).

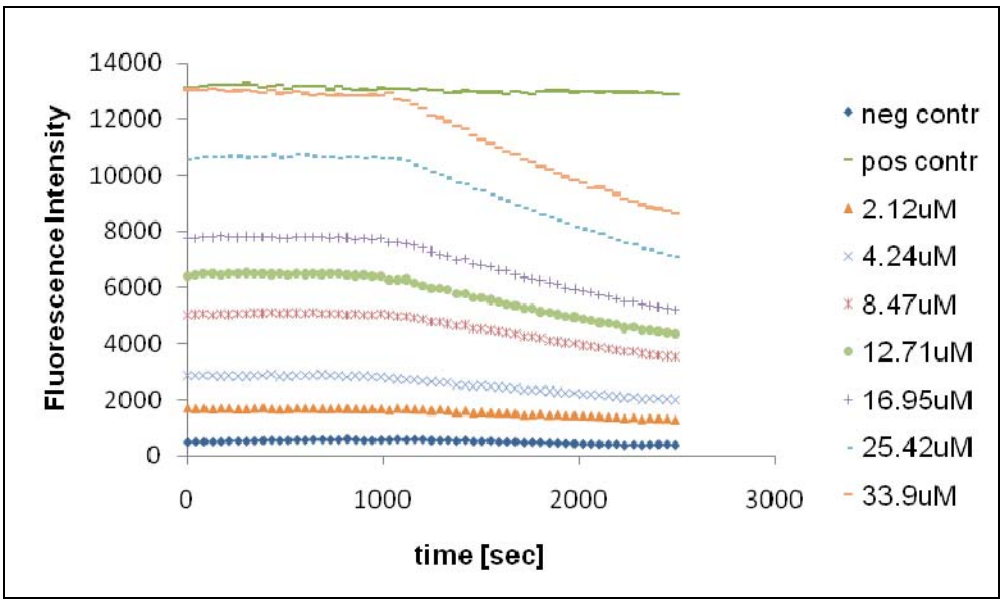


Fig. S16 Fluorimetric assay of ADP-ribosyl cyclase (ADPRC) and **3e**: **ADPR-cyclase activity**. *Conditions*: enzyme (0.075U/mL), **3e** (2.12-33.9µM), HEPES buffer (50mM, pH 7.4), 26 ºC, λ_{ex} 300nm, λ_{em} 410nm, gain 15%. Enzyme addition at 1030sec. Positive control: **3e** (33.9µM). Negative control: buffer.

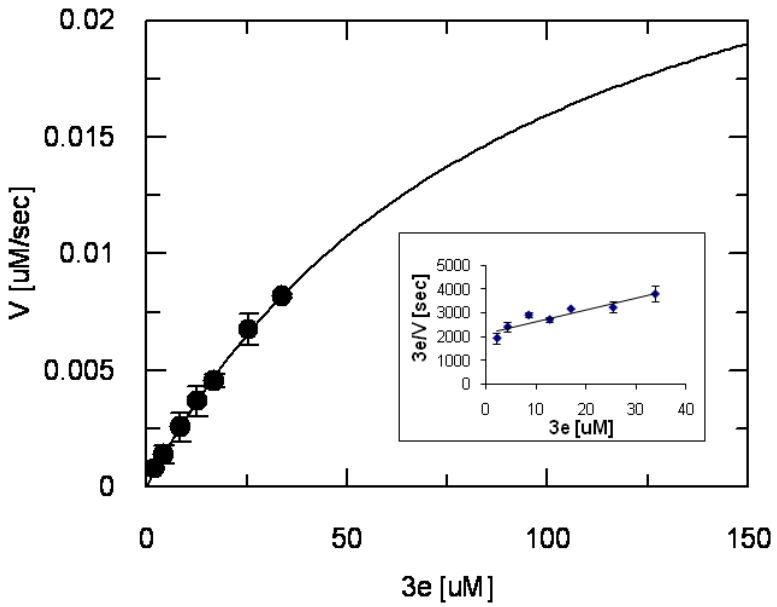
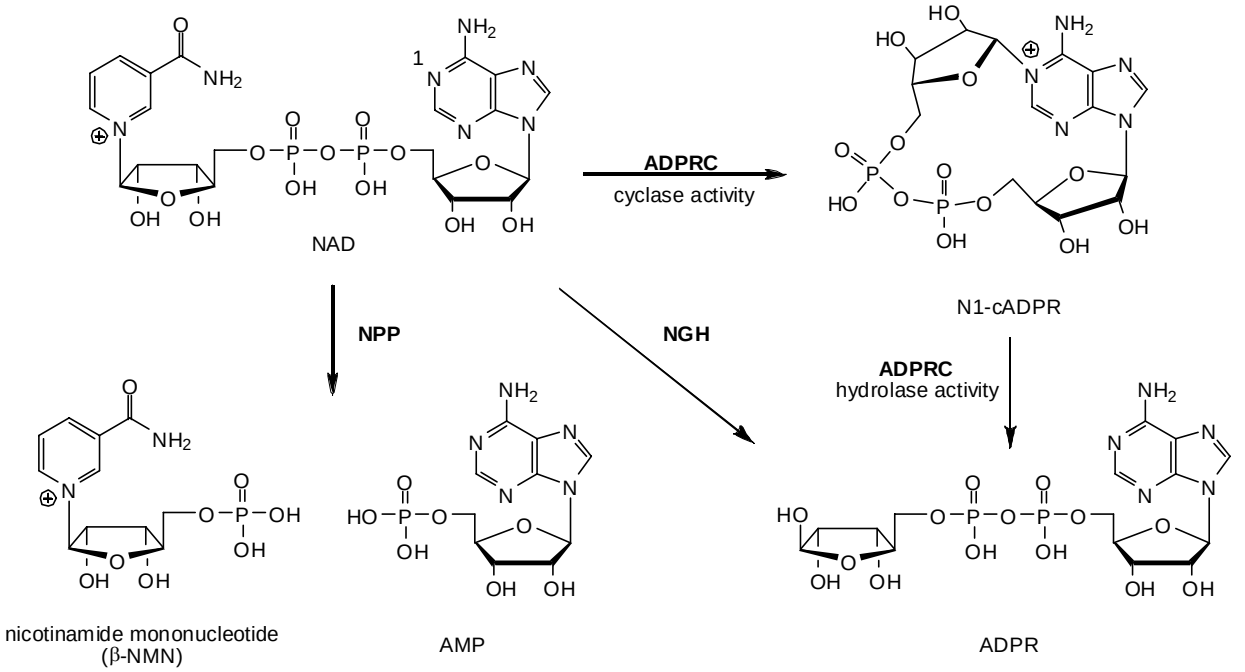
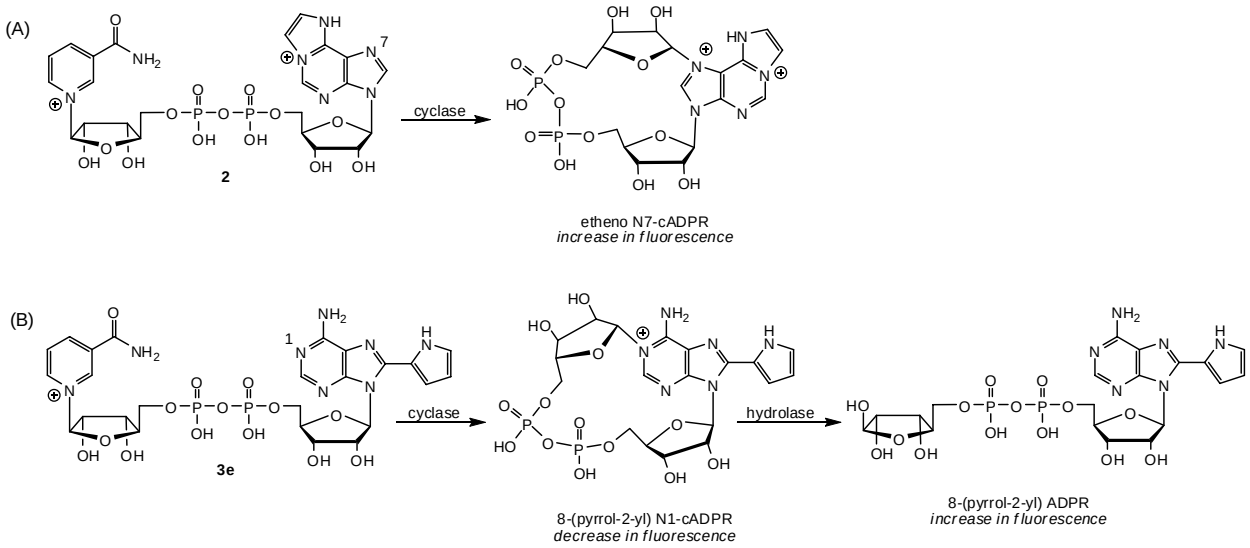


Fig. S17 Fluorimetric assay of ADP-ribosyl cyclase (ADPRC) and **3e**: **Michaelis-Menten and Hanes-Woolf plot (insert)**. *Conditions*: enzyme (0.075U/mL), **3e** (2.12-33.9μM), HEPES buffer (50mM, pH 7.4), 26ºC, λ_{ex} 300nm, λ_{em} 410nm, gain 15%. Initial velocity values were calculated from the respective reaction time course by measuring the fluorescence signal at 10% product formation. Initial velocity values were fitted to the Michaelis-Menten plot (with Grafit5) and to the Hanes-Woolf plot (with Excel, estimated linear equation y = 49.03x + 2154.6, r^2 = 0.8678).



Scheme S1 Reactions catalyzed by NAD-consuming enzymes used in this study. *NPP*: nucleotide pyrophosphatase (NPP) from *Crotalus adamanteus* venom [1], *NGH*: NAD-glycohydrolase (NGH) from porcine brain [2], *ADPRC*: ADP-ribosyl cyclase (ADPRC) from *Aplysia californica* [3]. ADPRC is a multifunctional enzyme that catalyzes primarily the N1-cyclization of NAD to cADPR (cyclic adenosine diphosphate ribose), but also the hydrolysis of cADPR to ADPR (adenosine diphosphate ribose) [3a].



Scheme S2 Reaction pathways for *Aplysia californica* ADPRC with alternative substrates **2** and **3e**. (**A)** ε-NAD **2** is cyclised in position N7. The resulting etheno N7-cADPR is more fluorescent than **2** [3b]. **(B) 3e** is cyclised, putatively, in position N1, like the natural substrate NAD. The resulting 8-(pyrrol-2-yl) N1-cADPR is less fluorescent than **3e**. At high enzyme concentrations, ADPRC catalyses the hydrolysis of 8-(pyrrol-2-yl) N1-cADPR into 8-(pyrrol-2-yl) ADPR, which leads to an increase in fluorescence.

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[3] (a) C. Cakir-Kiefer, H. Muller-Steffner, F. Schuber, *Biochem. J.* **2000**, *349*, 203; (b) R. M. Graeff, T. F. Walseth, H. K. Hill, H. C. Lee, *Biochemistry* **1996**, *35*, 379.