Enzymatic syntheis and biological use of photolabile nicotinic acid adenine dinuclotide phosopate (NAADP)

Raman Parkesh*, Sridhar R. Vasudevan, Alex Berry, Anthony Galione,
James Dowden and Grant C. Churchill*.
Department of Pharmacology, University of Oxford, Mansfield Road, Oxford, OX1 3QT
raman.parkesh@pharm.ox.ac.uk; grant.churchill@pharm.ox.ac.uk

Synthetic Methods and Materials: All Chemicals were purchased from Aldrich and used as received. All anhydrous solvents were either purchased in sure seal bottles or dried by standard procedure. Deutrated solvents were dried over 3 A^0 molecular sieves prior to use. ADP-ribosyl cyclase was obtained from Prof. Hong-Chung Lee, University of Minnesota. All untreated solvents used were of HPLC grade. ^{1}H NMR spectra were collected in $CDCl_3$ on a Bruker DQX-200, DPX-400 and AV-500 spectrometer at the Chemistry NMR Facility at the University of Oxford, Oxford. All chemical shifts are reported in the standard δ notation of parts per million using the peak of residual proton and carbon signals of $CDCl_3$ as internal reference. High-resolution mass spectral analyses were carried out at the Chemistry Mass Spectrometry Facility at the University of Oxford, Oxford.

Synthesis of 4,5-dimethoxy-2-nitroacetphenylhydrazone: 4,5-dimethoxy-2-nitroacetophenone (3.4 g, 15.10 mmol) was dissolved in 100 mL of ethanol. To this was added CH_3COOH (2.80 g, 46.7 mmol). The mixture was stirred for 15 minutes at room temperature and hydrazine monohydrate (3.12g, 2.68 mmol) was added. The resulting solution was refluxed for 3 hours. After cooling the room temperature, solution was diluted with 200 mL of deionized water and placed in an ice-bath. The compound seprated as yellow needles. The product was filtered, washed with ether and dried under vacuum. ^{1}H NMR (D_2O, 200 MHz): δ 7.61 (s, 1H), 6.87 (s, 1H), 5.39 (bs, 2H) 3.97 (s, 6H), 2.04 (s, 3H). ES-MS (M^+) Calculated: 223.227, Found: 223.226.

Synthesis of Caged NADP: The synthesis of NADP was followed from literature procedure with modifications.[1] NADP (100 mg, 0.152 mmol) was dissolved in 2 ml of deionized water in a 5 mL

reaction vial and the pH of the solution was adjusted to 4 with 1.0 M NaOH. 4,5-dimethoxy-2-nitroacetophenylhydrazone (0.301g, 1.337 mmol) was stirred in 2 mL of $CHCl_3$ in a separate reaction vial for 5 minutes and then freshly prepared[2] MnO_2 (0.807 g, 8.38 mmol) was added and the mixture stirred for 30 minutes. The resulting 1-(4,5-dimethoxy-2-nitropheyl) diazoethane was not isolated but was directly filtered through celite into the stirring NADP solution. These manipulations were performed in subdued light. The biphasic mixture was stirred vigoursly in dark for 2 hours. After this period, the $CHCl_3$ layer was removed, and one more addition of diazoethane was made. This biphasic mixture was then stirred overnight. The organic phase was removed and aqueous layer washed with $CHCl_3$ (3 x 5 mL) and evaporated. The product was purified by HPLC using AG MP-1 resin eluting with a linear gradient of 150 mmol Trifluoroacetic acid. The caged NADP eluted at 15 min. The fractions were collected and the evaporated on a rota vap. The reside was then dissolved in 1 mL of water and precipitated by addition of cold MeOH (-20 ^{0}C). The compounds show the expected peaks as reported earlier. ^{1}H NMR (D_2O, 200 MHz): δ 9.05 (bs, 1H), 8.77 (s, 1H), 8.58 (d, *J* =7.5 Hz, 1H), 8.08 (d, *J* = 12.8 Hz, 1H),7.91 (t, *J* = 7.1 Hz, 1H), 7.69 (d, *J* = 12.3 Hz, 1H), 7.09 (s, 1H), 6.67 (d, *J* = 10.5, 2H), 5.78 (d, *J* = 4.9 Hz, 1H), 5.58 (m, 2H), 4.98 (m, 1H), 4.38 (m, 1H), 4.25 (m, 1H), 4.20 (m, 2H), 4.12 (m, 1H), 4.02–3.95 (m, 4H), 3.65 (m, 6H), 1.21 (d, *J* = 6.1, 3H). 31P NMR (D2O with 50 mM NaOAC, pH 5.8, 162 MHz): δ -1.51, -11.10 (A-B, *J* = 20.4).

Enzymatic Synthesis of Caged NAADP: Caged NADP (10 mg, .0011 mM), Nicotinic acid (0.009mg, .0094 mmol) were dissolved in 10 mM aqueous AcOH/NaOH (pH 4, 1 mL). This solution was incubated with 10 µL of ADP-ribosyl cyclase at room temperature. After 3 h, HPLC analysis (AG MP-1, 150 mmol TFA) showed complete consumption of caged NADP and formation of caged NAADP (RT = 25.4 mins). The reaction mixture was further kept for 2 hours at room temperature. The crude mixture was purified by HPLC using AG MP-1 resin and eluting with aqueous TFA gradient (150 mM), the product eluted at 15 % TFA. The fractions were combined fractions and evaporated under vacuum at 50 ^{0}C. The residue wad dissolved in 1 ml of water and the compound precipitated by addition of cold methanol (-20 ^{0}C) as a light yellow powder. ^{1}H-NMR (400 MHz, D_2O): δ 9.32 (bs, 1H), 9.17 (d, *J*=5.9

Hz, 1H), 8.90 (d, *J*=8.1 Hz, 1H), 8.45 (s, 1H),8.28 (s, 1H), 7.60 (d, *J*= 12.0 Hz, 2H), 7.20 (s, 1H), 6.90 (d, *J*=10.5, 2H), 5.83 (m, 2H), 5.40 (m, 2 H), 4.97 (m, 1H), 4.50 (m, 1H), 4.44 (m, 1H;), 4.41 (m, 1H), 4.30 (m, 1H), 4.27–4.10(m, 4H), 3.75 (m, 6H), 1.40 (d, *J*= 6.1, 3H). ^{31}P NMR (D2O with 10 mM HEPES, pH = 7.4, 202.4 MHz) δ -2.7, -11.5.

Chromatographic Analysis: HPLC seperation was performed with columns (omnifit) packed with AG MP-resin and eluted with a nonlinear gradient of trifluoroacetic acid similar to that described previously.[2] Final purification of the caged NAADP was achieved using Mono Q columns using a gradient of water and triethyamine bicarbonate as reported previously.[2]

Ca^{2+} release in Intact Eggs: All the imaging was done using laser-scanning confocal microscope (Zeiss Axiovert) with excitation at 488 nm and emission collected above 505 nm selected with a long-pass filter. Lytechinus pictus eggs were used for the microinjection experiments. Sear urchin eggs were processed for the outer jelly layer to be removed. The dejellied eggs were then transferred to polylysine-coated glass cover slips, pressure microinjected with Oregon Green 488 BAPTA Dextran and DMNPE-caged NAADP.

After 10 minutes following the injection, DMNPE-caged NAADP was photolysed with an ultraviolet laser. Images were later processed with the software Image J (version 10.2) to create a self-ratio by dividing each image (F) by an image acquired before simulation (F_0). To confirm that the calcium increase was due to the DMNPE-caged NAADP, eggs were co-injected with sub-threshold amounts of free NAADP along with the caged compound. Following 10 minutes of incubation, uncaging was done. This does not cause an further increase in calcium indicating that Ca^{2+} increase are indeed due to NAADP and not the photolysed by products.

References:

1. Lee, H. C.; Aarhus, R..; Gee, K. R..; Kestner, T. *J. Biol. Chem.* **1997**, *272*, 4172–4178.

2. Cohen, B. E.; Stoddard, B. L.; Koshland, D. E., Jr. *Biochemistry* **1997**, *39*, 9035-9044.