Chemo-enzymatic synthesis and biological evaluation of photolabile nicotinic acid adenine dinuclotide phosphate ($NAADP^+$)†

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A chemo-enzymatic synthesis of novel caged $NAADP^+$ without the formation of multiple cage compounds has been achieved. The biological activity of the caged $NAADP^+$ was demonstrated by its fast uncaging in intact sea-urchin eggs.

$NAADP^+$, first identified as a calcium-releasing messenger in sea-urchin eggs,[1] is a structural analogue of nicotinamide adenine dinuclotide phosphate ($NADP^+$) in which the amide group is replaced with a carboxylic acid group. Interestingly, this minor structural variation makes $NAADP^+$ the most potent Ca^{2+}-mobilizing messenger.[1,2] It is understood that the carboxylate, amino and 2′-phosphate groups are crucial for $NAADP^+$-induced Ca^{2+} release, as several structural analogues of $NAADP^+$ in which these groups are modified show no effect on intracellular Ca^{2+} release.[3,4] The 2′-phosphate is particularly crucial for Ca^{2+} release, as $NAAD^+$ which lacks this group has no Ca^{2+}-mobilizing property.[3,4] Its mode of Ca^{2+} release is distinct from cyclic ADP-ribose (cADPR) and inositol 1,4,5-trisphosphate.

$NAADP^+$ operates on Ca^{2+} stores that have been identified as acidic lysosome-related organelles,[5] and recent studies have demonstrated the calcium-mobilizing efficiency of $NAADP^+$ in a wide variety of cells, from plant to animal, including humans.[6] However, $NAADP^+$ signalling transduction, the receptors it acts on and other regulatory functions are not clear. To fully understand the molecular basis underlying the $NAADP^+$ signalling process, it is necessary to deliver it intracellularly in a controlled fashion. In this context, blocking the active functionalities in $NAADP^+$ and unblocking them when necessary is promising. Cell-permeant photolabile caged $NAADP^+$ is the ideal molecular tool for investigating the localized signalling behavior of $NAADP^+$. To date there is only one report of caged $NAADP^+$, based on the caging reagent 1-(2-nitrophenyl)diazoethane (NPE), synthesized by direct alkylation of $NAADP^+$ at pH 1.3.[7] These extremely acidic conditions were used to avoid multiple caging of $NAADP^+$, but in spite of this precaution, significant caging of carboxylate groups with NPE was observed. The unstable nature of most caging reagents at low pH hindered the development of other caged $NAADP^+$ derivatives.

In the present study, we report the chemo-enzymatic synthesis and biological investigation of a novel caged $NAADP^+$ (4,5-dimethoxy-2-nitroacetophenone (DMNPE)–$NAADP^+$, Scheme 1). The presence of electron-donating methoxy groups on the caging chromophore should lead to fast release of $NAADP^+$. The effect of structural variations on the photocleavage kinetics of the nitrophenyl chromophore is well documented.[8–10] Furuta *et al.*[11] and Ellis-Davies *et al.*[12] have reported that DMNB-caged compounds uncage faster than simple *ortho*-nitrobenzyl-caged compounds in biological studies of photocaged molecules. Our attempts to directly cage $NAADP^+$ under a variety of conditions all proved unsuccessful, yielding only the starting material and a hydrolysis product, ADP-ribose phosphate. Initially, the highly labile and photosensitive diazo caging reagent **1** was prepared by the oxidation of 4,5-dimethoxy-2-nitroacetophenone with MnO_2 in $CHCl_3$. The diazo compound was then added to NADP under biphasic conditions (H_2O–$CHCl_3$) with vigorous stirring, resulting in the formation of caged $NADP^+$.[13] Finally, ADP-ribosyl cyclase catalyzed a base-exchange reaction between caged $NADP^+$ and excess nicotinic acid, resulting in the formation of DMNPE-caged $NAADP^+$ (Scheme 1). The negligible hydrolyzing activity of this enzyme prevented formation of side-products, resulting in the formation of caged $NAADP^+$ in higher yield.[14] The caging reaction was monitored by anion exchange high-performance liquid chromatography (HPLC). Final purification of DMNPE-caged $NAADP^+$ was achieved by HPLC using AG MP-1 columns (see ESI†).

The structure of DMNPE-caged $NAADP^+$ was confirmed by both ^{1}H-NMR and ^{31}P-NMR. The ^{1}H-NMR of the purified compound showed two additional resonance peaks in the aromatic region, the H3 and H6 of the phenyl. Morever, the methoxy groups at C4 and C5 are singlets, while the benzylic methyl is a doublet. The ^{31}P-NMR of DMNPE-caged $NAADP^+$ showed only two peaks at −2.08 ppm and −11.31 ppm (see ESI†), which correspond to the 2′-phosphate and pyrophosphate groups. The 2′-phosphate is shifted upfield by about 2 ppm compared to that of $NAADP^+$, while the pyrophosphate signal is practically unchanged. This dramatic shift is consistent with the caging group being attached to the 2′-phosphate. As reported by Lee *et al.*,[7] we also observed peak broadening, which might be due to intermolecular hydrogen bond formation. We believe that the hydrophobic nature of the caging group will favour intermolecular association, thus restricting the molecular motion of the caged compound. The high concentration of the sample (5 mg ml^{-1}) needed to acquire the ^{31}P-NMR spectra further promotes these intermolecular and π–π stacking interactions.

The quantum yield (Φ) of the DMNPE-caged $NAADP^+$ measured by comparison with a known standard, 4-methyl-7-nitroindolinylglutamate, was found to be 0.20 at pH 7. Both the

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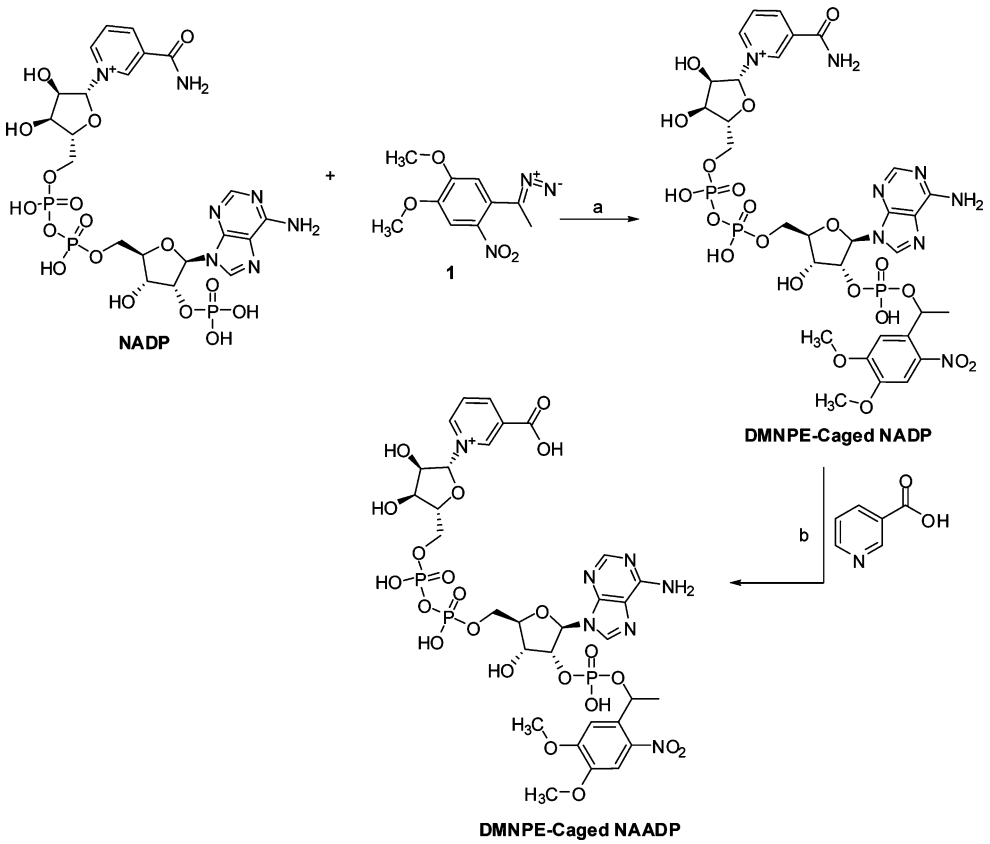

Scheme 1 Chemical structure and synthesis of DMNPE-caged NAADP$^+$. *Reagents and conditions*: (a) $CHCl_3$–H_2O, pH 4, 14 h, 60%; (b) ADP-ribosyl cyclase, acetate buffer pH 4.4, 5 h, 80%.

DMNPE-caged NAADP$^+$ and the standard were photolysed separately to check that there was no interference from simultaneous photolysis. DMNPE-caged NAADP$^+$ has an extinction coefficient (ε) of 18 000 and 8900 M^{-1} cm^{-1} at 250 nm and 350 nm respectively. This value is comparable to the other DMNPE-caged compounds.

The photolysis of DMNPE-caged NAADP$^+$ to NAADP$^+$ was confirmed by HPLC (see ESI†). The columns were packed with AG MP-1 resin and eluted with non-linear gradient of trifluoroacetic acid (150 mM). The HPLC trace of DMNPE-caged NAADP$^+$ showed a major peak with a retention time of 25.4 minutes. Upon photoirradiation, DMNPE-caged NAADP$^+$ showed a new peak with a retention time of 21.2 minutes, identical to that of NAADP$^+$ (Fig. 1).

The sea-urchin was used as a model system for studying the Ca^{2+} response of caged NAADP$^+$ because its large size (100 μm) facilitates microinjection. The sea-urchin eggs contain robust Ca^{2+}-release channels for the NAADP$^+$ signalling system, making it the most reliable biological system for directly studying Ca^{2+} release from intracellular stores. The ability of DMNPE-caged NAADP$^+$ to mobilize Ca^{2+} in intact sea-urchin eggs is depicted in Fig. 2. It is well-known that even nanomolar concentrations of NAADP$^+$ are sufficient to bind the receptor and block the calcium response.[1,7,15] To avoid this desensitization of the receptor, caged NAADP$^+$ was pretreated with alkaline phosphate[7] to remove any trace amounts of free NAADP$^+$.

Calcium release was monitored by 488 BAPTA Dextran Oregon Green, co-injected into the eggs along with DMNPE-caged NAADP$^+$. Exposure of the eggs to UV light resulted in an increase

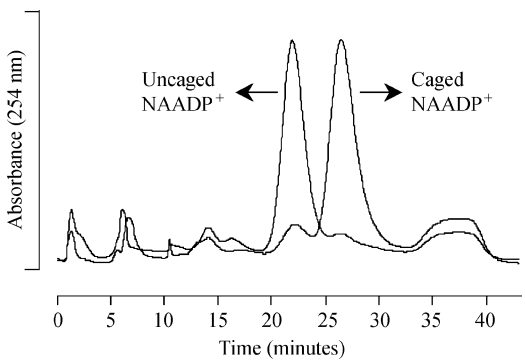

Fig. 1 HPLC chromatogram of the DMNPE-caged NAADP$^+$ and uncaged NAADP$^+$. As can be seen, the uncaging reaction is quite facile, and leads to a quantitative production of NAADP$^+$ (left-hand trace).

in Ca^{2+} consistent with NAADP$^+$ release. In another experiment, eggs were injected with caged NAADP$^+$ containing a small amount of NAADP$^+$ (10 nM) to inactivate the response. No Ca^{2+} was observed after UV irradiation. This conforms that the calcium response is indeed due to the NAADP$^+$ released from the cage.

In conclusion, this report describes the novel chemo-enzymatic synthesis of caged NAADP$^+$ without the formation of multiple cages. Biological evaluation in sea-urchin eggs demonstrated the fast release of DMNPE-caged NAADP$^+$. The mild enzymatic route (pH > 4) will allow the caging of NAADP$^+$ with novel caging chromophores such as two-photon active reagents. Further studies

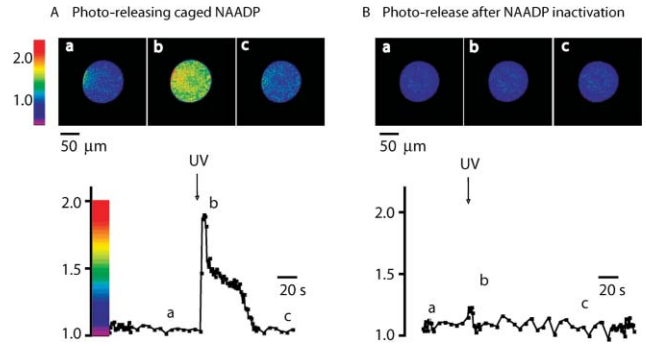

Fig. 2 (A) Effect of photoreleasing DMNPE-caged NAADP$^+$ on Ca^{2+} signalling in intact sea-urchin eggs. (B) Injecting sub-threshold amounts of NAADP$^+$ (10 nM), prior to photoreleasing DMNPE-caged NAADP$^+$, failed to elicit a response, suggesting the inactivation of NAADP$^+$ receptors. Images are self-ratio, with colours representing the ratio according to the calibration scale. Traces are of average ratio over times. Eggs contained Oregon Green 488 BAPTA Dextran (10 µM) and DMNPE-caged NAADP$^+$ (0.5 µM) as labelled. Caged NAADP$^+$ was photoreleased as indicated by the arrows.

to measure the two-photon absorption cross-sectional area and its biological application are currently underway.

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