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Nicotinic Acid Adenine Dinucleotide Phosphate Analogs Substituted on the Nicotinic Acid and Adenine Ribosides. Effects on Receptor-Mediated Ca^{2+} release

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Abstract

Nicotinic acid adenine dinucleotide phosphate (NAADP) is a Ca^{2+} releasing intracellular second messenger in both mammals and echinoderms. We report that large functionalized substituents introduced at the nicotinic acid 5-position are recognized by the sea urchin receptor, albeit with a 20–500 fold loss in agonist potency. 5-(3-Azidopropyl)-NAADP was shown to release Ca^{2+} with an EC_{50} of 31 μM and to compete with NAADP for receptor binding with an IC_{50} of 56 nM. Attachment of charged groups to the nicotinic acid of NAADP is associated with loss of activity, suggesting that the nicotinate riboside moiety is recognized as a neutral zwitterion. Substituents (Br^- and N_3^-) can be introduced at the 8-adenosyl position of NAADP while preserving high potency and agonist efficacy and an NAADP derivative substituted at both the 5-position of the nicotinic acid and at the 8-adenosyl position was also recognized although the agonist potency was significantly reduced.

INTRODUCTION

Stimulation of Ca^{2+} release from intracellular stores is accomplished through increases in the intracellular concentrations of second messengers such as D-myo-inositol trisphosphate (IP_3), cyclic ADP-ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP, **1**) which interact with specific receptors to regulate Ca^{2+} selective ion-channels.

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ASSOCIATED CONTENT

Supporting Information. ^1H NMR spectra, results from combustion analysis, HPLC results for tested dinucleotides, and UV absorption spectra for tested dinucleotides are presented in Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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In response to extracellular stimuli, these messenger molecules are released, bind to their specific receptors, and initiate complex changes in the intracellular Ca^{2+} concentration, ultimately converting extracellular stimulation into a highly regulated spatiotemporal physiological signal.^{1,2,3} Of the Ca^{2+} releasing second messengers, NAADP is the most potent ($\text{EC}_{50} = 20 \text{ nM}$ in sea urchin egg homogenates), yet it remains the least characterized. Originally discovered as a contaminant in commercial preparations of NADP,⁴ NAADP regulates receptor-mediated Ca^{2+} release from intracellular Ca^{2+} stores distinct from those regulated by either IP3 or cADPR.⁵ NAADP-mediated Ca^{2+} signaling is best defined in sea urchin eggs, but has been demonstrated in a variety of mammalian and human systems including pancreatic cells,^{2,6,7} brain,⁸ heart,^{9,10,11,12} kidney cells,¹³ lymphocytes,^{14,15,16} and the human cell line Jurkat^{17,18} among others.^{19,20,21,22} Although NAADP has been known for 2 decades,^{4,23,24} and despite its importance in a variety of cell signaling pathways, information concerning structural features of NAADP important for its recognition is incomplete. The lack of information is, in part, due to the complexity of its structure which prohibits facile synthesis and testing of structurally modified analogs. Various reports have defined the importance of charge, location and size of functional groups necessary for NAADP recognition by its receptor.^{25,26,27} The 3-nicotinyl carboxylic acid, 2'-phosphate and the 6-adenosyl amine were all recognized as required for activity. Removal of these functionalities, as well as changing their location resulted in diminished or in complete loss of activity. More recently, the effect of substitution at the 4- and 5-nicotinic acid positions of **1** was evaluated.²⁸ When nicotinic acid modified NAADP analogs were tested using the sea urchin NAADP receptor, small chemical substitutions present at the 5-nicotinyl position (amino, methyl, ethyl, azido) were found to be well tolerated, whereas substitution at the 4-nicotinyl position (amino, methyl, butyl, phenyl) were shown to be low potency agonists.

The receptor which binds NAADP and controls Ca^{2+} release has not been identified. The two pore channel (TPC) was proposed to be a specific Ca^{2+} channel regulated by NAADP,²⁹ but this has recently been challenged.^{30,31} Photoaffinity studies using [³²P]-5-N₃-NAADP have identified 45, 40, 35 kDa proteins in sea urchin homogenates and 21/22 kDa proteins in cultured human cells that are photolabeled specifically. These NAADP binding proteins may be important in the regulation of Ca^{2+} release.^{32,33}

The development of chemical probes that can be used to identify the NAADP binding proteins requires the production of more highly functionalized NAADP analogs than those reported in our previous publication.²⁸ Receptor identification will require high potency analogs containing functional groups suitable for ligand-affinity chromatography, or bifunctional derivatives suitable for covalent labeling of the receptor followed by affinity isolation of the derivatized protein using “click chemistry”. This work describes an extension of our study of the structure activity relationships (SAR) of NAADP derivatives to 11 additional NAADP analogs, five of which contain large substituents containing functional groups at the nicotinic acid 5-position. We also evaluate the effect of substitution on the 8-adenosyl position and for the first time report the activity of a disubstituted NAADP derivative modified at both the 5-nicotinyl and 8-adenosyl positions.

RESULTS

Chemistry

Substituted NAADP analogs that would otherwise be difficult to prepare using purely synthetic methodologies can be produced from a synthetic pyridine base and NADP using the *Aplysia californica* ADP-ribosyl cyclase³⁴ (a version of NAD glycohydrolase).^{35,36} Illustrated in Figure 1, the enzyme catalyzed base-exchange reaction was instrumental in producing the substituted NAADP derivatives which were used in this study. In the presence of NADP (or an 8-substituted NADP), and a high concentration of 5-substituted nicotinic acid (**R₁**), the *Aplysia* ADP-ribosyl cyclase catalyzes the base exchange of nicotinamide for the 5-substituted nicotinic acid. The chemoenzymatic synthesis, although powerful, is subject to the requirement that the pyridine base be an efficient substrate for the ADP-ribosyl cyclase. This is usually the case, but there are pyridine bases that fail to exchange.

Synthesis of 5-substituted nicotinic acids

Simple 5-substituted nicotinic acids such as 5-hydroxynicotinic acid (**2a**) and 5-bromonicotinic acid (**2b**) were available commercially. Others were produced in a single step from a commercially available compound. For example, 5-acetamidonicotinic acid (**2c**) was made by treatment of 5-aminonicotinic acid with acetic anhydride. 5-Thiomethyl nicotinic acid (**2d**) was derived from **2b** by nucleophilic aromatic displacement with sodium thiomethoxide.³⁷ In total, 10 nicotinic acid derivatives were obtained and evaluated as base-exchange substrates with the expectation of producing the corresponding NAADP analogs (Table 1).

A majority of the functionalized 5-substituted nicotinic acid analogs were synthesized using the Sonogashira reaction^{38,39} which effectively coupled a convenient starting material (5-bromonicotinic acid ethyl ester, (**2e**)) with various terminal alkynes. The resulting alkynyl nicotinic acid derivative was reduced by hydrogenation, further derivatized, and then hydrolyzed providing a series of 5-substituted nicotinic acid analogs. The synthesis of **2f** (Scheme 1) illustrates typical methodologies in the synthesis of 5-substituted pyridine carboxylic acids. First, Sonogashira reaction of 5-bromonicotinic acid ethyl ester with phenylacetylene in the presence of Pd(OAc)₂, triphenylphosphine and CuI yielded 5-(2-phenylethynyl)-3-pyridinecarboxylic acid ethyl ester. This alkyne was reduced to alkane by catalytic hydrogenation, and hydrolysis with NaOH provided carboxylic acid (**2f**).

The synthesis of 5-(3-acetamidopropyl)-nicotinic acid (**6**) is illustrated in Scheme 2. Alkyne **3**⁴⁰ was coupled to **2e** under Sonogashira coupling conditions. The product was then reduced by hydrogenation (**5**), and saponified with aqueous base providing acetamide **6**. Synthesis of 5-(3-aminopropyl)-nicotinic acid (**12**) was accomplished by coupling *t*-Boc-N protected propargyl amine (**7**)⁴¹ yielding alkyne **8** (Scheme 3). Standard hydrogenation conditions provided reduced compound **9**. Removal of the *t*-Boc protecting group with TFA yielded **10** followed by alkaline treatment to produce desired aliphatic amine **12**. Compound **9** was alternatively hydrolyzed yielding **11**.

Similarly, palladium catalyzed couplings with propargyl alcohol and **2e** (Scheme 4) provided a route to nicotinic acid derivatives **15** and **18**.

Synthesis of NAADP analogs containing 8-substituted adenosyl derivatives

In order to determine the effect of substitution within the purine ring of NAADP, we needed to produce 8-adenosyl substituted NAD analogs, convert these to NADP derivatives and next transform these into NAADP derivatives using the pyridine base exchange reaction as depicted in Figure 1. 8-N₃-adenosyl-NADP (**22**) was conveniently synthesized starting with 8-bromoadenosine⁴² according to Scheme 5. 8-Bromoadenosine was converted to 8-N₃-adenosine (**20**) in two steps,^{43,44} and **20** was selectively phosphorylated producing **21**.⁴⁵ The pyrophosphate bond was formed by coupling **21** with of commercially available nicotinamide mononucleotide yielding 8-azido-NAD (**22**).⁴⁶ Selective phosphorylation at the 2'-adenosyl position of **22** was accomplished enzymatically on a small scale with recombinant human NAD kinase and ATP providing 8-azido NADP (**23**).

8-Bromo-NADP (**24**) was synthesized directly from NADP by bromination in buffered water (Scheme 6). Conditions for the synthesis of 8-bromo-NAD^{47,48} and 8-bromo-AMP⁴⁹ have been previously reported, and NADP was similarly converted to **24**.⁵⁰

NAADP analogs

NAADP analogs **25–35** (Table 2) were synthesized using the enzyme catalyzed pyridine-base exchange reaction between a 5-substituted nicotinic acid (R₁) and either NADP or an 8-substituted NADP derivative as illustrated in Figure 1. Pyridine bases **2b** and **2f** failed as substrates in the enzymatic base exchange, and NAADP derivatives derived from these were therefore not synthesized. This illustrates a well know limitation of this chemoenzymatic synthesis, wherein electron deficient or water insoluble bases fail to exchange.

Spectroscopic properties of NAADP analogs

The electronic absorption spectrum of 5-hydroxy-NAADP (**26**) at low pH exhibits maxima at 256 and at 298 nm (Figure 2, panel A). This 298 nm band is not observed in the spectrum of NAADP and is due to the conjugation of the 5-oxosubstituent with the pyridinium ring. As the pH of the solution was increased, the long wavelength band shifts to longer wavelength and exhibits a λ_{max} at 333 at high pH. An analysis of the change in the absorption spectra of **26** as a function of pH (Figure 2, panel B) enabled us to determine that the pK_a for the ionization of the 5-OH of **26** was 5.85. The previously characterized 5-amino-NAADP also exhibits a long wavelength absorption maximum at 346 nm, but this band does not shift between pH 2.5 and 11.5, indicating that the less acidic –NH₂ group does not ionize below pH 11.5.

Biological Activity

NAADP analogs were evaluated using *Strongylocentrotus purpuratus* (sea urchin) egg homogenates, which serve as a model for NAADP mediated activity in mammalian cells and were used for much of the characterization of the cADPR and NAADP mediated Ca²⁺ release. The ability of NAADP analogs (**25–35**) to elicit Ca²⁺ release from sea urchin egg

homogenates was tested using a fluorometric assay. Microsomes were loaded with Ca^{2+} in the presence of ATP and 3 μM fluorescent indicator fluo-3. Upon addition of an NAADP analog, Ca^{2+} was released and its binding to the Ca^{2+} indicator results in an enhanced fluorescent signal. The increase in fluorescence was proportional to the released Ca^{2+} and can be measured using a fluorescence plate reader (excitation 490 nm and emission 535 nm). These experiments were performed in triplicate at a variety of agonist concentrations and EC_{50} values were determined (Table 3).

Competition ligand binding studies measured the ability of analogs to compete with radiolabeled [^{32}P]NAADP for receptor binding (Table 4). Binding studies were performed using 96-well filter plates. A constant concentration of [^{32}P]NAADP (0.2 nM) and varying concentrations of the test ligand and were incubated simultaneously (90 min) with sea urchin egg homogenates. After incubation, the homogenate was filtered, washed and radioactivity remaining on the filter determined by liquid scintillation. Seven concentrations of each analog were tested to determine IC_{50} values.

The sea urchin NAADP receptor was shown to be desensitized upon pretreatment with subthreshold concentrations of NAADP. Subsequent challenge with high concentrations of NAADP failed to release Ca^{2+} from intracellular stores. A final assay determines the ability of subthreshold concentrations of each analog to induce receptor desensitization. A 7 min pretreatment with varying concentrations of an NAADP analog (**25–35**) was followed by addition of saturating NAADP (1 μM) and fluorometric measurement of Ca^{2+} release. The response was reported as the IC_{50} value for inhibition of Ca^{2+} release (Table 5). Experiments were performed in triplicate.

Figure 3 illustrates typical concentration response relationships for NAADP and **32** for the above assays. Both compounds demonstrate high potency for the sea urchin NAADP receptor. The IC_{50} for competition ligand binding and the EC_{50} for sub-threshold desensitization were roughly equal, and both of these values were approximately 100-fold lower than the EC_{50} for Ca^{2+} release. This is in accord with previous studies that propose that the sea urchin NAADP receptor contains a high-affinity inhibitory and a low-affinity stimulatory binding sites.⁵¹ 5-Thiomethyl-NAADP (**32**) was shown to be a full agonist.

Figure 4 depicts the results of bioassay of 5-(3-azidopropyl)-NAADP (**31**). Analog **31** represents an NAADP derivative containing a large functionalized substituent. It released Ca^{2+} as a partial agonist at μM concentrations, approximately 1000-fold higher than **1**, but was recognized in the nM range in the competition ligand binding assay and in assays of subthreshold desensitization.

DISCUSSION

Chemoenzymatic synthesis enabled us to efficiently produce 11 new NAADP analogs, several of which extend our previously reported series in that they contained large, functionalized groups attached to the nicotinic acid ring.²⁸ Limitations of the chemoenzymatic synthesis were nonetheless evident, since **2b** and **2f** could not be converted into the corresponding NAADP derivatives. Failure of the enzymatic pyridine-base

exchange reaction could result from poor solubility of the pyridine base, low nucleophilicity of the pyridine nitrogen, or inability of the enzyme active site to accommodate the structure of the modified base.

In this work the scope of the chemoenzymatic synthesis was extended through the use of recombinant human NAD kinase for catalysis of the specific phosphorylation of the 2'-adenosine hydroxyl in an NAD derivative. Thus, 8-N₃-NAD (**22**) was converted to the NADP analog 8-N₃-NADP (**23**), which was further converted to 8-N₃-NAADP (**34**) by enzyme catalyzed base exchange. The use of NAD kinase enables us to extend the scope of the chemoenzymatic synthesis and utilize some of the available purine substituted NAD analogs as precursors to NAADP derivatives.

All of the NAADP analogs were tested for their ability to release Ca²⁺ ion, for their ability to compete with [³²P]NAADP in competition ligand binding, and for their ability to induce subthreshold receptor desensitization. The IC₅₀ values measured for competition ligand binding and for sub-threshold desensitization agree well with each other. These values are consistently 1–2 orders of magnitude lower than the EC₅₀ values for Ca²⁺ release. Previous characterization of the NAADP receptor in sea urchin egg extracts concluded that the receptor contained a high affinity inhibitory binding site and a low affinity stimulatory binding site.⁵¹ Our observations are consistent with this model, assuming that the IC₅₀ values measured using either competition ligand binding or receptor desensitization reflect the strength of interactions with the high affinity site whereas the Ca²⁺ release assay detects the low affinity stimulatory site.

In our previous publication,²⁸ NAADP analogs containing small substituents (eg. amino- and methyl-) at the 5-position of the nicotinic acid ring of NAADP (**1**) were shown to be agonists which were approximately equipotent with NAADP. In this study, 5-hydroxy-NAADP (**26**) demonstrated unexpected loss of potency relative to its bioisosteric analogs 5-methyl-NAADP and 5-amino-NAADP.²⁸ Introduction of the phenolic substituent (**26**) resulted in a 74.9 fold loss of potency compared to NAADP, whereas 5-amino-NAADP exhibited only a 1.5 fold decrease in potency. This curious difference was unexpected and can best be attributed to the ionization of the phenolic group at the pH used for bioassay. Bioassay for Ca²⁺ release was performed at pH 7.2, and with a phenolic pK_a of 5.85 compound **26** can be calculated to be 96% ionized. If unionized **26** is the form recognized at the receptor, at the observed EC₅₀ of 1932 nM, the concentration of unionized **26** will be 77 nM or 3-fold higher than NAADP (**1**). This suggests that the receptor requires the pyridinium functionality to be zwitterionic and to possess no net charge. This proposal was further supported by inactivity of 5-carboxy-NAADP²⁸ and the inactivity of **29** containing an ammonium group. The primary amine of **29** will be protonated at physiological pH, affording a net +1 charge on the pyridinium ring explaining the loss of potency when compared with similar analogs **27–31**. Based upon these results, we conclude that the pyridinium moiety must be zwitterionic and possess no net charge for receptor-mediated events to occur.

Thiomethyl compound (**32**) was a full agonist with an EC₅₀ for Ca²⁺ release 6-fold higher relative to NAADP (Table 3, Figure 3) and with IC₅₀ values of 18.4 and 8.9 fold increases

for competitive ligand binding and desensitization, respectively (Tables 4, 5). Additional small substitutions at the 5 position, exemplified by acetamido (**25**) were also shown to be high potency agonists. The slight loss of activity in **25** (EC_{50} = 24.9 fold increase), as well as its binding (IC_{50} = 42.9 fold increase for competitive binding, IC_{50} = 51.2 fold increase for desensitization) could be explained by the increased steric bulk.

A goal of this study was to develop functionalized NAADP analogs that retained high affinity for the receptor. Derivatives with a 3 carbon arm between the pyridine ring and various functional groups (**27–31**) were synthesized and evaluated. Both **27** and **28** are branched, and in the case of *t*-Boc derivative (**28**), contain a sterically large and hydrophobic group. The smallest member of this series, **30** exhibited about a two order of magnitude decrease in potency in all of our assays relative to NAADP. The largest member of the series, **28** was found to be a low-potency partial agonist. This derivative demonstrated a 3–4 order of magnitude loss of potency. The “clickable” azidopropyl analog (**31**) displayed interesting activity in that it was a partial agonist (Figure 4) active in the 30 μ M range in inducing Ca^{2+} release. Binding assays however (Tables 4 and 5) suggest that **31** binds with high potency to the inhibitory binding site with an EC_{50} of approximately 50 nM.

In this work we additionally reported that substituents could be introduced at the 8-adenosyl position while retaining agonist potency. The data in Tables 3–5 show that NAADP analogs with substituents at the 8-adenosyl position were recognized as high potency agonists. We found that 8-azido-NAADP (**34**) was a high potency agonist, as both the EC_{50} and IC_{50} values were no more than 2-fold higher than those measured for NAADP. 8-Bromo-NAADP (**33**) was shown to be less potent, with an EC_{50} 27.5 fold-higher than that of NAADP. Binding studies measured IC_{50} values to be 42.4 fold increase for competitive-ligand binding experiments and 32.6 fold higher than NAADP for desensitization. Together, the 8-adenosyl position has been identified as a position tolerant to substitution. This is significant, because previously reported modification of the adenine ring was associated with loss of activity.²⁶

A disubstituted NAADP derivative has been synthesized and tested in the sea urchin egg system (Tables 3–5). 5-Azido-8-bromo-NAADP (**35**) was a low potency partial agonist, exhibiting IC_{50} and EC_{50} values 1000 fold higher than NAADP. Analog **35** was less potent than predicted by the activities of either of the mono-substituted analogs, indicating that the presence of substituents at both the 8-adenosyl and the 5-nicotinic acid interfere with binding. Nonetheless, disubstituted **35** was bound at sufficiently low concentration (370–576 nM) to suggest that derivatives with substituents on both the nicotinic acid 5-position and on the 8-adenosyl position can be developed and applied to receptor identification.

We recently reported the results of a study of the activities of several NAADP analogs in the SKBR3 human cell line.³⁷ We found that the SAR for nicotinic acid substitution differs significantly from the relation we report here based on sea urchin egg homogenate. In SKBR3 cells, 5-thiomethyl-NAADP (**32**) loses much of its efficacy, and in the cases tested the 4-substituted analogs are better tolerated than are the 5-substituted analogs. This indicated that the details of the SAR for recognition of NAADP analogs will differ across species.

CONCLUSIONS

We found that 5-substituted nicotinic acids containing large and functionalized substituents were excellent substrates for the enzyme catalyzed base-exchange reaction. Similarly, we find that 8-adenosyl substituted NADP derivatives can be prepared from NAD analogs and serve as substrates for base-exchange. This significantly extends the scope of our chemo-enzymatic method. The corresponding NAADP derivatives were evaluated in sea urchin egg homogenates for their binding, for their ability to induce sub-threshold desensitization and for Ca^{2+} release activity. The results confirm that small substitutions, as seen with analogs **25**, **26** and **32**, were high potency agonists. Hydroxypropyl (**30**) and azidopropyl (**31**) NAADP analogs demonstrated relatively high activity. The loss of potency associated with the *t*-Boc derivative **28**, was informative, indicating the upper limit of steric bulk permitted on the 5-nicotinyl position. We found that compounds containing substitution on the 8-adenosyl position were potent agonists, in particular azide **34**, which was found to be almost equipotent to NAADP itself. Another novel observation was the ability of a disubstituted NAADP derivative to elicit a receptor-mediated response at low concentrations. This finding will prove to be extremely valuable for the development of NAADP derivatives for the purification and the identification of the unknown NAADP receptor using photoaffinity labeling and “click chemistry” affinity isolation. In addition to further elucidation of the SAR, we have also confirmed the observation that the pyridinium moiety on NAADP must be zwitterionic and possess no net charge on this functionality to be recognized by the NAADP receptor.

EXPERIMENTAL SECTION

Materials

Reagents were obtained from either Acros Organics or Sigma-Aldrich, and purified when necessary according to instructions in Perrin's Purification of Laboratory Chemicals.⁵² Anhydrous solvents were purchased from Sigma-Aldrich (St. Louis, MO) or purified by distillation over a drying agent as described in the individual experimental description. All reagent grade solvents (acetone, DCM, methanol, ethyl acetate (EtOAc), and hexanes) were purchased from Pharmco-Aaper or EMD chemicals. 5-Aminonicotinic acid was purchased from AK Scientific (Union City, CA), 5-hydroxynicotinic acid was purchased from TCI America (Portland, OR), NAD was purchased from Roche Diagnostics (Indianapolis, IN) and NADP monosodium salt, tetra-hydrate was purchased from Research Products International (Mt. Prospect, IL). 5-Thiomethylpyridine-3-carboxylic acid (5-thiomethylnicotinic acid, **2d**) was synthesized according to the procedure described by Ali et al.³⁷

Recombinant *Aplysia californica* ADP-ribosyl cyclase was produced by the procedure of H. C. Lee et al.⁵⁰ Concentrated solutions of the purified enzyme were stored at -80°C in aliquots of 100 μL at a concentration of 10 mg/mL. As the catalyst was needed for synthesis, a single 100 μL aliquot was removed from the freezer and thawed to 0°C in an ice-bucket. This concentrate was diluted to a final working concentration of 0.2 mg protein/mL by diluting it into 4.9 mL of 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (pH = 7.5). The sample was mixed thoroughly and divided into 60 μL

aliquots each containing 12 μg of protein at a concentration of 200 $\mu\text{g}/\text{mL}$. Normally, 3 aliquots (36 μg of protein) were used for base exchange of 18 μmol of NADP. Diluted samples are stored at $-80\text{ }^{\circ}\text{C}$ and are warmed to $0\text{ }^{\circ}\text{C}$ in an ice bath immediately prior to use.

General procedures

Reactions were performed in washed and oven-dried glassware ($110\text{ }^{\circ}\text{C}$) under an inert atmosphere of dry nitrogen gas. Reactions were stirred using a magnetic stirring apparatus with Teflon-coated stir bars. Microwave heating of reactions was performed using a Biotage Initiator 2.5 system and the conditions were reported by describing the instrument's adjustable settings: time, temperature, pre-stirring, vial type, absorption level, and fixed hold time. The vials, caps and stir bars for this system were purchased from Biotage.

Determination of pH was made using a PHM82 Standard pH Meter (Radiometer America, Cleveland OH).

Evaporation of volatile solvents was done under reduced pressure using a Heidolph rotary evaporator and a pressure regulator connected to either a water aspirator or a polytetrafluoroethylene (PTFE) diaphragm vacuum pump. In experimental descriptions, reduced pressures in mbar are often stated. For less volatile solvents, a traditional 2-stage oil-driven vacuum pump connected to a Buchi rotary evaporator and fitted with a heating bath was used.

Melting points were determined in duplicate on a MEL-TEMP II apparatus and are uncorrected. Elemental analyses were performed by Atlantic Microlabs (Norcross, GA) and were regarded as being acceptable when the result was within $\pm 0.4\%$ of the theoretical values. Compounds reported as solvates are denoted on an individual basis, and the reported empirical formula was calculated by incorporating the minimum amount of the designated solvent that the compound encountered during purification in order to be within the acceptable range ($\pm 0.4\%$).

Proton (^1H) NMR and carbon (^{13}C) NMR were determined using either a Unity-400 spectrophotometer (400 MHz), a Varian Inova-600 spectrophotometer (600 MHz), or a Bruker Avance Cryprobe (600 MHz). Chemical shifts are reported in ppm (δ) and were referenced to the residual proton signal of the deuterated solvent. When TMS was present (CDCl_3 and deuterated dimethylsulfoxide (d_6 -DMSO)), the TMS peak at zero ppm was used as the reference. ^{31}P NMR spectra were recorded on a Unity-400 spectrophotometer (162 MHz) and referenced externally to 85% H_3PO_4 . The chemical shifts for ^1H NMR were reported to the second decimal place while ^{13}C chemical shifts were reported to the first decimal place. The following abbreviations are used to describe spin multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets. Coupling constants (J) were reported in Hertz (Hz).

Mass spectral analysis of substituted nicotinic acid derivatives and dinucleotides were performed at The Ohio State University Mass Spectrometry & Proteomics Facility. Accurate mass analysis was performed using ESI ionization in the positive ion mode (ESI^+), and dinucleotides were measured using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry.

Analytical thin layer chromatography (TLC) was performed on Baker-flex TLC plates (2.5 × 7.5 cm) with a 254 nm fluorescent indicator (IB-F). Plates were developed in a covered chamber, usually with 5–10 mL of mobile phase, and visualized by UV-light. Flash chromatography was performed according to the procedure of Still et al.⁵³ using Fisher Silica Gel 60, 200–425 mesh (40–60 mm) weighing about 50 times the weight of the crude mixture to be purified. Alternatively, flash chromatography could be accomplished using a Combiflash Rf system (Teledyne-Isco, Lincoln, NE). Prepacked columns containing normal phase silica columns (12 g or 40 g) and reversed-phase C₁₈-columns (15.5 g) were purchased from Teledyne-Isco for this apparatus. Linear gradients could be more reproducibly produced by the Combiflash Rf system and often provides a superior result.

Reverse phase C18 columns for the Teledyne-Isco Combiflash (Lincoln, NE) and were used to further purify the substituted nicotinic acids and as a desalting procedure. The method consisted of applying the dinucleotide as a solution in water followed by applying a gradient formed from 0–80 % methanol in water. The product generally elutes before the gradient starts and the rest of the method can be considered a wash and storage procedure.

High pressure liquid chromatography (HPLC)

HPLC purifications were performed on a Bio-Rad BioLogic Duo Flow system equipped with a 254 nm UV-detector. Analytical anion-exchange columns were purchased from Bio-Rad (Uno-Q, 7 × 35 mm, 1.3 mL). Alternately, both analytical and preparative columns could be packed from BioRex MP1-X2 analytical anion exchange resin. The procedure was adapted from the volatile water-TFA system described by Alexson et al. (1981).⁵⁴

Preparation of macroporous anion exchange resin—Bio-Rad AG-MP1 anion exchange resin (30 g) (Bio-Rad Laboratories, Hercules, CA USA) was added to a stirred solution of 4 N NaOH (200 mL) and thoroughly mixed for 6 h. Stirring was stopped and the resin was allowed to settle overnight, at which time, excess liquid was decanted and a second portion of 2 N NaOH (200 mL) was added and stirred for 3 h. Once the resin settled, excess liquid was decanted and then resuspended and stirred (30 min) in water (250 mL). After decanting, the resin was washed twice more with water as above, for a total of three water washes. We needed to monitor the presence of chloride anion during the washes with hydroxide anion, since it is the hydroxide anion that displaces the chloride anion. A portion of resin free supernatant (1 mL) was added to a test tube, acidified with concentrated aqueous nitric acid (7 drops), and then assayed with silver nitrate. If chloride ion was present, insoluble silver chloride precipitates out of solution. The first two washes with NaOH indicated Cl[−] was present, while the third was Cl[−] free, indicating that displacement of Cl[−] with OH[−] was complete. Washings with water were then conducted until supernatants are silver chloride negative and the pH of the water wash was neutral. If silver chloride continues to precipitate out of solution, the procedure must be repeated to appropriately convert the resin to the [−]OH form. Upon testing negative for silver chloride, the resin was decanted and resuspended in 2 M TFA (150 mL). This final mixture is stirred for 30 min before storage at 4 °C and was ready for use.

Method 1: Analytical method—A Bio-Rad Uno-Q column (7 × 35 mm, 1.3 mL of anion-exchange resin) was fitted to a Bio-Rad BioLogic Duoflow HPLC apparatus, equipped with a 250 µL injection loop. The flow rate was 3 mL/min throughout the separation. The injection loop was completely filled with solution of the NAADP derivative (1 mg/mL), and the chromatography begun by applying 1 mL of d•H₂O through the injection loop. The separation was developed by 1) applying 10 mL of d•H₂O; 2) forming a linear gradient between d•H₂O and 50 mM aqueous TFA over a total volume of 60 mL; 3) applying 11 mL of 100 mM aqueous TFA. Finally, the column was re-equilibrated with d•H₂O (11 mL) in preparation for the next injection.

Method 2: Analytical method—The conditions were identical to those described under Method 1, except that an injection loop and an injection volume of 50 µL was used and the chromatography begun by applying 0.8 mL of d•H₂O through the injection loop. Thereafter, the chromatography was developed by 1) applying 9 mL of d•H₂O; 2) forming a linear gradient between d•H₂O and 15 mM aqueous TFA over a total volume of 120 mL; 3) applying 9 mL of 100 mM aqueous TFA. Finally, the column was re-equilibrated with d•H₂O (12 mL) in preparation for the next injection.

Method 3: Prep-scale HPLC (0–50 mM TFA)—A glass column (Omni, 1.5 × 11.5 cm) filled with Bio-Rad AG-MP1 ion exchange resin (trifluoroacetate form, see resin preparations) was connected to a Bio-Rad BioLogic Duoflow HPLC apparatus fitted with a 5 mL injection loop. The flow rate throughout was 5 mL/min. The injection loop was completely filled with a solution of the NAADP derivative (1 to 3 mg/mL), and the chromatography begun by applying 7 mL of d•H₂O through the injection loop. The preparative separation was developed by 1) applying 20 mL of H₂O; 2) forming a linear gradient between H₂O and 50 mM aqueous TFA, formed over a total volume of 160 mL; 3) applying 25 mL of 100 mM aqueous TFA. Finally, the column was re-equilibrated with d•H₂O (35 mL). Peaks absorbing at 254 nm were combined and excess TFA was removed by extraction of the aqueous solution with DCM (3×). Following the extraction, residual DCM and TFA were removed by treatment of the aqueous solution under vacuum for 10 min at 25 mbar.

Method 4: Prep-Scale HPLC (0–20 mM aqueous TFA)—The procedure was identical to that described under Method 3, except that the chromatography was begun by applying 9 mL of d•H₂O through the injection loop and developed by 1) applying 15 mL of d•H₂O; 2) forming a linear gradient between d•H₂O and 20 mM aqueous TFA over a total volume of 225 mL; 3) applying 40 mL of 100 mM aqueous TFA. Finally, the column was re-equilibrated with d•H₂O (40 mL) in preparation for the next injection.

Method 5: Prep-Scale HPLC (0–8 mM aqueous TFA)—The procedure was identical to that described under Method 3, except that the chromatography was begun by applying 9 mL of d•H₂O through the injection loop and the separation developed by 1) applying 15 mL of d•H₂O; 2) forming a linear gradient between d•H₂O and 8 mM aqueous TFA over a total volume of 190 mL; 3) applying 40 mL of 100 mM aqueous TFA. Finally, the column was re-equilibrated with d•H₂O (40 mL) in preparation for the next injection.

Open Column Anion-Exchange Chromatography

Open-column anion exchange chromatography for the purification of dinucleotides was performed using diethylaminoethyl (DEAE) substituted cellulose (DE53, product #4058200; Whatman Inc, Florham Park, NJ USA). To prepare the commercially obtained material for use, 50 g of DEAE cellulose was added to a stirred solution of 1.5 M NH_4HCO_3 (300 mL) and thoroughly mixed for 4 h. Stirring was stopped and the resin was allowed to settle overnight, at which time, excess liquid was decanted and a second portion of 1.0 M NH_4HCO_3 (250 mL) was added and stirred for 2 h. Once the resin settled, excess liquid was decanted the resin re-suspended and stirred (30 min) in water (300 mL). After decanting, the resin was washed twice more with water as above, for a total of three water washes. At this point, the pH of a sample of the supernatant should be close to neutral, even slightly acidic as measured using a wide-range pH test paper. Repeated washes with water are continued until the pH is neutral. Unused resin that is not to be used within 2 weeks was stored at 4 °C in 100 mM NH_4HCO_3 .

Method 6: Desalting of NAADP derivatives on DEAE cellulose—Pyridine dinucleotides **25–35** were desalted and obtained in the ammonium form using a simple gradient in a volatile NH_4HCO_3 buffer followed by repeated lyophilization. A Bio-Rad Econo-Column (2.5 × 8 cm) was filled with a slurry of DE53 cellulose and allowed to form a packed column of absorbent 3 cm high. Water (25 mL) was passed through the column until the effluent pH was neutral. The sample of dinucleotide was applied to the column as a dilute solution at pH 7.5 and properly adsorbed. The separation was developed by applying a linear gradient formed between water (150 mL) and 0.5 M NH_4HCO_3 (150 mL). Application of a gradient (0–0.5 M NH_4HCO_3) allows proper binding of the dinucleotide to the column. A flow rate of about 1–2 mL/min is achieved using a peristaltic pump to produce a slight positive pressure. Contaminating salts such as NaCl, ammonium chloride, or sodium trifluoroacetate elute into the low ionic strength buffer ahead of the dinucleotide. NAADP derivatives generally elute into the mobile phase at concentrations of $\text{NH}_4\text{HCO}_3 > 200$ mM. Fractions are collected automatically with the aid of a fraction collector (Teledyne Isco, Retriever 500, 100 drops/tube) and the dinucleotide detected by its absorption at 254 nm. Repeated lyophilization of the sample from water ensures complete removal of volatile NH_4HCO_3 , leaving pure, salt free dinucleotide.

Method 7: Preparative Anion exchange chromatography on columns of DEAE Cellulose—NAADP derivatives can be separated from NADP, ADP-ribose phosphate, and other nucleotides by anion exchange chromatography on DEAE cellulose. DE-52 cellulose was slurry packed into glass Bio-Rad Econo-Columns (2.5 × 50 cm) to form a resin bed approximately 42 cm high. Water (100 mL) was passed through the column to ensure the pH of the effluent from the column was neutral. The dinucleotide mixture was applied to the column as a dilute solution at pH 7.5, and washed into the bed with 10–20 mL of distilled water. The separation was developed by the application of a linear gradient formed between water (350 mL) and 0.6 M NH_4HCO_3 (350 mL). A flow rate of about 1–2 mL/min is achieved using a peristaltic pump (Teledyne-Isco, TRIS) to produce a slight positive pressure. The separation allows purification of compounds based on charge and NAADP derivatives usually elute into the middle of the gradient, into approximately 320 mM

NH₄HCO₃. Fractions were collected using an automatic fraction collector (Teledyne- Isco, Retriever 500, 200 drops/tube), and the dinucleotides detected by their UV absorbance (ISCO UA-6 UV/Vis Detector). UV absorbing peaks were combined, frozen, and lyophilized. Repeated lyophilization of the sample from water ensures complete removal of volatile NH₄HCO₃, leaving pure, salt free dinucleotide.

5-(Acetamido)nicotinic acid (2c)

5-Aminonicotinic acid (0.250 g, 1.81 mmol), acetic anhydride (1.11 g, 11 mmol) and pyridine (0.72 g, 10 mmol) were added to a round bottom flask and refluxed for 22 h. According to the original procedure, a precipitate should form and this was recrystallized from ethanol. This did not work in our hands. Instead, solvents were removed by distillation *in vacuo*. The sample was dissolved in DMSO and purified by column chromatography on silica gel in butanol-acetic acid-water (5:2:3). This gave a mixture of two compounds. The sample was further purified by column chromatography on silica gel (90:9:1 DCM-methanol-acetic acid). Removal of solvents by distillation under reduced pressure afforded a dark yellow solid (126 mg, 39%): mp 277–279 °C (lit.⁵⁵ 282–284 °C). TLC R_f 0.73 in butanolacetic acid-water (5:2:3), TLC R_f 0.36 in DCM-methanol-acetic acid (90:9:1; the R_f value was recorded following 2 developments of the TLC plate). ¹H NMR (400 MHz, *d*₆-DMSO) δ 10.37 (bs, 1H), 8.88 (s, 1H), 8.74 (s, 1H), 8.58 (s, 1H), 2.11 (s, 3H).

5-Bromonicotinic acid ethyl ester (2e)

To a solution of 5-bromonicotinic acid (5 g, 28.4 mmol) in absolute ethanol (50 mL) was added 5 N HCl in dioxane (10 mL). The reaction mixture was refluxed for 48 h. Solvents were removed *in vacuo* then water (75 mL) was added and neutralized with 5 N NaOH. Upon neutralization, the solution was extracted 3× with EtOAc. EtOAc was distilled under reduced pressure to give an off-white solid which was recrystallized twice from water affording a white solid (5.7 g, 88%): mp 39–40 °C (lit.⁵⁶ 38–39 °C). TLC R_f 0.52 in 20% EtOAc-hexanes. ¹H NMR (600 MHz, CDCl₃) δ 9.13 (s, 1H), 8.84 (s, 1H), 8.43 (s, 1H), 4.43 (q, 2H, *J* = 7.2 Hz), 1.42 (t, 3H, *J* = 7.2 Hz). ¹³C NMR (101 MHz, CDCl₃) δ 164.0, 154.4, 148.8, 139.5, 127.6, 120.6, 62.0, 14.2.

5-(2-Phenylethyl)-3-pyridinecarboxylic acid (2f) was synthesized in three steps starting from **2e**.

5-(2-Phenylethynyl)nicotinic acid ethyl ester

To a solution of **2e** (250 mg, 1.09 mmol), Pd(OAc)₂ (12 mg, 0.054 mmol) and DIPEA (423 mg, 3.27 mmol) and triphenylphosphine (14 mg, 0.054 mmol) in acetonitrile (7.5 mL) was added phenylacetylene (134 mg, 1.31 mmol) and CuI (14.5 mg, 0.0763 mmol) while stirring under N₂. After the addition is complete, the reaction mixture was refluxed for 16 h. At this time, TLC indicated that the starting material was consumed. Solvents were removed *in vacuo* and the residue partitioned between water and chloroform. The phases were separated and chloroform was concentrated *in vacuo*. The resulting oil was purified by column chromatography on silica gel (20% EtOAc-hexanes) to yield a brown oil (224 mg, 82%): TLC R_f 0.48 in 20% EtOAc-hexanes. ¹H NMR (600 MHz, CDCl₃) δ 9.14 (d, 1H, *J* = 2 Hz), 8.90 (d, 1H, *J* = 2 Hz), 8.41 (t, 1H, *J* = 2 Hz), 7.56 (m, 2H), 7.39 (m, 3H), 4.44 (q, 2H, *J* =

7.14 Hz), 1.43 (t, 3H, $J = 7.14$ Hz). ^{13}C NMR (150 MHz, CDCl_3) δ 164.7, 155.3, 149.3, 139.3, 131.8, 129.11, 128.52, 125.9, 122.1, 120.5, 93.7, 85.0, 61.7, 14.3. Anal. ($\text{C}_{16}\text{H}_{13}\text{NO}_2$) C, H, N.

5-(2-Phenylethyl)nicotinic acid ethyl ester

5-(2-Phenylethynyl)nicotinic acid ethyl ester (209 mg, 0.832 mmol) was dissolved in EtOAc (12 mL) and methanol (4 mL) and transferred to a glass hydrogenation flask and cooled in dry ice-acetone for 5 min. Pd/C (42 mg, 20 % w/w) was added and the flask was attached to a PARR hydrogenator and shaken for 10 h at 30 PSI of H_2 . Upon completion, the mixture was filtered through Celite and solvents were removed from the filtrate *in vacuo*. The resulting residue partitioned between water and chloroform. The chloroform was concentrated under reduced pressure and the product was purified by column chromatography on silica gel (50% EtOAc-hexanes) to yield a yellow oil (175 mg, 82%): TLC R_f 0.54 in 50% EtOAc-hexanes. ^1H NMR (600 MHz, CDCl_3) δ 9.06 (d, 1H, $J = 2$ Hz), 8.53 (d, 1H, $J = 2.2$ Hz), 8.10 (t, 1H, $J = 2$ Hz), 7.29 (m, 2H), 7.22 (m, 1H), 7.15 (m, 2H), 4.41 (q, 2H, $J = 7.14$ Hz), 2.98 (m, 4H), 1.41 (t, 3H, $J = 7.14$ Hz). ^{13}C NMR (150 MHz, CDCl_3) δ 165.4, 153.3, 148.4, 140.3, 137.08, 136.80, 128.57, 128.46, 126.39, 126.06, 61.5, 37.2, 34.7, 14.3. Anal. ($\text{C}_{16}\text{H}_{17}\text{NO}_2$) C, H, N.

5-(2-Phenylethyl)-3-pyridinecarboxylic acid (2f)

5-(2-Phenylethyl)nicotinic acid ethyl ester (127 mg, 0.497 mmol) was dissolved in methanol (3 mL) and 1 N NaOH (3 mL) and stirred at rt for 18 h. After this time the methanol was evaporated *in vacuo* and the remaining solution was neutralized using 1 N HCl. A cream-colored precipitate formed, which was collected and washed with ice cold water (20 mL). The solid was dried overnight in a vacuum desiccator at 40 °C equipped to an oil-driven vacuum pump to yield a cream-colored solid (80 mg, 71%): mp 212–214 °C. TLC R_f 0.71 in DCM-methanol-acetic acid (94:5:1). ^1H NMR (600 MHz, d_6 -DMSO) δ 13.37 (s, 1H), 8.89 (d, 1H, $J = 2$ Hz), 8.62 (d, 1H, $J = 2.2$ Hz), 8.12 (t, 1H, $J = 2$ Hz), 7.27 (m, 2H), 7.23 (m, 2H), 7.18 (m, 1H), 3.12 (m, 4H). ^{13}C NMR (150 MHz, d_6 -DMSO) δ 166.3, 153.4, 147.8, 140.7, 136.82, 136.51, 128.38, 128.17, 126.02, 125.89, 36.3, 33.5. Anal. ($\text{C}_{14}\text{H}_{13}\text{NO}_2$) C, H, N.

3-Acetamidoprop-1-yne (3)

To a solution of propargylamine (500 mg, 9.1 mmol) and TEA (1.37 g, 13.7 mmol) in DCM (5 mL) at 0 °C was added acetic anhydride (1.11 g, 11 mmol). The mixture was stirred for 1 h at 0 °C, then left stirring for an additional 23 h at rt. Solvent was distilled *in vacuo* and then the product was purified by bulb-to-bulb distillation (0.3 mm Hg, 110–130 °C) affording a clear oil which was precipitated with hexanes, the precipitate collected, and recrystallized from ether-DCM to afford white crystals (510 mg, 58 %): mp 77–78 °C (Lit.⁴⁰ 83–85 °C). ^1H NMR (400 MHz, CDCl_3) δ 5.91 (s, 1H), 4.05 (m, 2H), 2.24 (s, 1H), 2.02 (s, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 169.7, 79.5, 71.6, 29.2, 23.0.

5-(3-Acetamidoprop-1-yne)nicotinic acid ethyl ester (4)

To a solution of **2e** (250 mg, 1.09 mmol), Pd(PPh₃)₂Cl₂ (38 mg, 0.0545 mmol) and DIPEA (423 mg, 3.27 mmol) in anhydrous acetonitrile (5 mL) were added **13** (127 mg, 1.31 mmol) and CuI (15 mg, 0.0763 mmol) while stirring under N₂. After the addition is complete, the reaction mixture was refluxed for 10 h. At the end of this time TLC indicated that the starting material was consumed. Solvents were removed *in vacuo* and the residue partitioned between water and chloroform. The chloroform was distilled *in vacuo* and the residue was purified by column chromatography on silica gel (0–5 % DCM-methanol) affording a yellow solid (161 mg, 60%). To obtain analytically pure samples, the yellow solid was recrystallized from ether-DCM affording a white powder: mp 82–84 °C. TLC R_f 0.63 in 5% methanol-DCM. ¹H NMR (400 MHz, CDCl₃) δ 9.11 (s, 1H), 8.78 (s, 1H), 8.29 (s, 1H), 6.82 (s, 1H), 4.42 (q, 2H, *J* = 7.2 Hz), 4.32 (d, 2H, *J* = 5.2 Hz), 2.08 (s, 3H), 1.41 (t, 3H, *J* = 7.2 Hz); ¹³C NMR (101 MHz, CDCl₃) δ 170.0, 164.5, 155.3, 149.4, 139.6, 125.9, 119.8, 90.0, 78.7, 61.8, 29.8, 23.0, 14.2. Anal. (C₁₃H₁₄N₂O₃) C, H, N. HRMS calcd for C₁₃H₁₄N₂O₃: 269.0902 (M+Na). Found *m/z*: 269.0891 (M+Na).

5-(3-Acetamidopropyl)nicotinic acid ethyl ester (5)

Compound **4** (260 mg, 1.06 mmol) was dissolved in methanol (7 mL) and transferred to a Pyrex hydrogenation flask and cooled in dry ice-acetone for 5 min. Pd/C (52 mg, 20% w/w) was added, then the flask was attached to a PARR hydrogenator and shaken for 8 hrs at 30 PSI of H₂. Upon completion, the mixture was filtered through Celite and solvent was distilled *in vacuo*, and the residue then partitioned between water and chloroform. The phases were separated and the chloroform was distilled *in vacuo*. This residue was purified by column chromatography on silica gel (0–10% methanol- DCM) to yield a white powder (240 mg, 91%): mp 50–52 °C. TLC R_f 0.55 in 10% methanol- DCM. ¹H NMR (400 MHz, CD₃OD) δ 8.92 (s, 1H), 8.61 (s, 1H), 8.23 (s, 1H), 4.40 (q, 2H, *J* = 7.2 Hz), 3.21 (t, 2H, *J* = 6.8 Hz), 2.75 (t, 2H, *J* = 8 Hz), 1.94 (s, 3H), 1.84 (m, 2H), 1.40 (t, 3H, *J* = 7.2 Hz). ¹³C NMR (101 MHz, CD₃OD) δ 173.3, 166.4, 154.2, 148.8, 139.5, 138.6, 128.0, 62.8, 40.0, 31.8, 31.0, 22.7, 14.7. HRMS calcd for C₁₃H₁₈N₂O₃ 273.1215 (M+Na). Found *m/z*: 273.1215 (M+Na).

5-(3-Acetamidopropyl)nicotinic acid (6)

Compound **5** (104 mg, 0.42 mmol) was dissolved in methanol (2 mL) and 4N NaOH (2 mL). This mixture was stirred at rt for 3.5 h. The solvent was removed under reduced pressure and the residue extracted 5 × with hot methanol (10 mL portions). The resulting 50 mL extract was filtered through Celite, and the solvent removed from the filtrate by distillation *in vacuo*. The yellow powder was purified by reverse phase chromatography by using a C₁₈ cartridge (15.5 g, isocratic elution with water) using Combiflash Rf system. The product eluted shortly after the void volume (13.5 mL), which upon removal of solvent *in vacuo* afforded an oily white solid (83 mg, 89%): mp 268–270 °C. TLC R_f 0.53 in butanol-acetic acid-water (5:2:3). ¹H NMR (400 MHz, CD₃OD) δ 8.89 (s, 1H), 8.42 (s, 1H), 8.18 (s, 1H), 3.22 (t, 2H, *J* = 6 Hz), 2.72 (t, 2H, *J* = 7.6 Hz), 1.94 (s, 3H), 1.85 (m, 2H). ¹³C NMR (150 MHz, CD₃OD) δ 173.4, 172.8, 151.1, 149.0, 138.69, 138.58, 135.0, 40.0, 31.8, 31.1, 22.7. HRMS calcd for C₁₁H₁₄N₂O₃: 245.0902 (M+Na). Found *m/z*: 245.0893 (M+Na).

tert-Butyl-prop-2-ynylcarbamate (7)

To a solution of propargylamine (750 mg, 14.0 mmol) in DCM (5 mL) at 0 °C was added a solution of di-*tert*-butyldicarbonate (3.36 g, 15.4 mmol) in DCM (6 mL) over a period of 20 min. After this 20 min period, the solution was allowed to warm to rt and stirred for an additional 48 h. Solvent was distilled *in vacuo* and the resulting yellow oil was stored at 4 °C for 16 h. A yellowish-white precipitate formed which was collected, dried under reduced pressure, and recrystallized from ether-DCM affording white crystals (1.5 g, 56%): mp 37–39 °C (lit.⁴⁰ 34–36 °C). ¹H NMR (400 MHz, CDCl₃) δ 4.79 (s, 1H), 3.93 (s, 2H), 2.22 (s, 1H), 1.46 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 155.4, 80.27, 80.05, 71.3, 30.4, 28.4.

5-[3-[(*tert*-Butyloxy)carbonyl]amino]propyn-1-yl]nicotinic acid ethyl ester (8)

To a solution of **2e** (250 mg, 1.09 mmol), Pd(PPh₃)₂Cl₂ (38 mg, 0.0545 mmol) and anhydrous DIPEA (423 mg, 3.27 mmol) in acetonitrile was added **14** (203 mg, 1.31 mmol) and CuI (15 mg, 0.0763 mmol) while stirring under N₂. After the addition was complete, the reaction mixture was refluxed for 6 h. After this time, TLC indicated complete consumption of starting material. The solvent was removed by distillation *in vacuo* and the residue partitioned between DCM and water. The phases were separated and the organic phase distilled under reduced pressure. The resulting residue was purified by column chromatography on silica gel (0–33% EtOAc-hexanes) affording a yellow oil which upon freezing at –20 °C for 16 h produced a yellowish-white solid (202 mg, 61%): mp 54–56 °C. TLC R_f 0.52 in 50% EtOAc-hexanes. ¹H NMR (400 MHz, CDCl₃) δ 9.12 (s, 1H), 8.79 (s, 1H), 8.30 (s, 1H), 5.52 (s, 1H) 4.42 (q, 2H, *J* = 7.2 Hz), 4.21 (s, 2H), 1.48 (s, 9H), 1.41 (t, 3H, *J* = 7.2 Hz). ¹³C NMR (101 MHz, CDCl₃) δ 164.5, 155.4, 149.4, 139.5, 125.8, 119.9, 90.5, 80.0, 78.6, 61.7, 31.0, 28.4, 28.0. Anal. (C₁₆H₂₀N₂O₄•0.1CH₂Cl₂) C, H, N. HRMS calcd for C₁₆H₂₀N₂O₄: 327.1321 (M+Na). Found *m/z*: 327.1315 (M+Na).

5-[3-[(*tert*-Butyloxy)carbonyl]aminopropyl]nicotinic acid ethyl ester (9)

Compound **8** (100 mg, 0.346 mmol) was dissolved in methanol (5 mL) and transferred to a Pyrex hydrogenation flask, which was capped and cooled in dry ice-acetone for 5 min. Pd/C (20 mg, 20 % w/w) was added and mixed and the flask was connected to a PARR hydrogenator and shaken for 6 h at 35 PSI of H₂. Upon completion, the mixture was filtered through Celite. The solvent was removed by distillation *in vacuo* and the residue partitioned between water and DCM. The phases were separated and the DCM was concentrated *in vacuo*. The resulting residue was purified by column chromatography on silica gel (50% EtOAc-hexanes), which upon distillation of the solvents *in vacuo* afforded a yellowish-white solid (99 mg, 93%): TLC R_f 0.31 (50% EtOAc-hexanes). mp 59–60 °C. ¹H NMR (400 MHz, CDCl₃) δ 9.06 (s, 1H), 8.61 (s, 1H), 8.13 (s, 1H), 5.07 (s, 1H), 4.42 (q, 2H, *J* = 7.2 Hz), 3.21 (q, 2H, *J* = 6.4 Hz), 2.73 (t, 2H, *J* = 8 Hz), 1.87 (m, 2H), 1.45 (s, 9H), 1.42 (t, 3H, *J* = 7.2 Hz). ¹³C NMR (101 MHz, CDCl₃) δ 165.4, 156.0, 153.4, 148.5, 136.8, 136.6, 126.0, 79.2, 61.4, 40.0, 31.4, 30.0, 28.4, 14.3. HRMS calcd for C₁₆H₂₄N₂O₄: 331.1634 (M+Na). Found *m/z*: 331.1646 (M+Na).

5-(3-Aminopropyl)nicotinic acid ethyl ester (10)

Compound **9** (97.5 mg, 0.316 mmol) was dissolved in water (1 mL) and TFA (1 mL) and stirred for 2 h at 37 °C. The solvent was removed *in vacuo* forming a yellow colored oil: TLC R_f 0.67 in butanol-acetic acid-water (5:2:3); R_f 0.12 in DCM-methanol-acetic acid (90:9:1). ^1H NMR (400 MHz, CD_3OD) δ 9.17 (s, 1H), 8.90 (s, 1H), 8.77 (s, 1H), 4.47 (q, 2H, $J = 7.2$), 3.05 (t, 2H, $J = 7.2$ Hz), 2.99 (t, 2H, $J = 8$ Hz), 2.08 (m, 2H), 1.42 (t, 3H, $J = 7.2$ Hz). ^{13}C NMR (101 MHz, CD_3OD) δ 164.3, 148.5, 144.7, 144.4, 141.7, 130.5, 63.7, 40.0, 30.3, 29.5, 14.6. The compound was immediately taken to the next step in the formation of **12**.

5-[3-[(*tert*-Butyloxy)carbonyl]aminopropyl]nicotinic acid (11)

Compound **9** (100 mg, 0.324 mmol) was dissolved in methanol (2 mL) and 4 N NaOH (1 mL) and stirred at 37 °C for 4 h. After this time, the solvent was removed and the product was dried *in vacuo* then neutralized by dissolving in 1 N HCl. The water was removed by lyophilization then the product was immediately purified by column chromatography on silica gel (90:9:1 DCM-methanol-acetic acid) affording a yellowish-white solid (81 mg, 89%): mp 225–227 °C. TLC R_f 0.47 in DCM-methanol-acetic acid (90:9:1); R_f 0.79 in butanol: acetic acid: water (5:2:3). ^1H NMR (400 MHz, CD_3OD) δ 8.89 (s, 1H), 8.41 (s, 1H), 8.17 (s, 1H), 3.10 (t, 2H, $J = 6.4$ Hz), 2.71 (t, 2H, $J = 8$ Hz), 1.82 (m, 2H), 1.43 (s, 9H). ^{13}C NMR (150 MHz, CD_3OD) δ 172.8, 158.6, 151.1, 148.9, 138.8, 138.7, 134.9, 80.0, 40.9, 32.5, 31.0, 28.9. Anal. ($\text{C}_{14}\text{H}_{20}\text{N}_2\text{O}_4 \cdot 0.5$ DCM) C, H, N.

5-(3-Aminopropyl)nicotinic acid (12)

Compound **9** (100 mg, 0.324 mmol) was dissolved in water (2 mL) and TFA (1 mL) and was stirred at rt for 8 h. The solvents were evaporated *in vacuo*. The crude residue was examined and NMR verified the removal of the *t*-Boc group, while the ethyl ester was intact. The sample was then dissolved in methanol (4 mL) and 4 N NaOH (2 mL) and the mixture allowed to stir at rt for 8 h. The reaction mixture was neutralized with 1 N HCl and solvents were removed *in vacuo*. The relatively pure mixture which resulted was further purified by reverse phase chromatography on a C_{18} cartridge (15.5 g, 13.5 mL void volume) using a Combiflash R_f system. The product eluted between column volumes 3 and 4 in an isocratic (water) system. Removal of water by lyophilization yielded a white solid (48 mg, 83%): mp 270–272 °C. TLC R_f 0.33 in butanol-acetic acid-water (5:2:3). ^1H NMR (600 MHz, D_2O) δ 8.59 (s, 1H), 8.23 (s, 1H), 7.87 (s, 1H), 2.9 (t, 2H, $J = 6.4$ Hz), 2.59 (t, 2H, $J = 8$ Hz), 1.85 (m, 2H). ^{13}C NMR (150 MHz, D_2O) δ 172.9, 150.2, 147.0, 137.2, 136.3, 132.0, 38.9, 28.8, 28.0. HRMS calcd for $\text{C}_9\text{H}_{12}\text{N}_2\text{O}_2$: 181.0977 (M+H), Found m/z : 181.0981 (M+H).

5-(3-Hydroxy-1-propynyl)nicotinic acid ethyl ester (13)

To a solution of **2e** (250 mg, 1.09 mmol), $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$ (38 mg, 0.0545 mmol) and DIPEA (423 mg, 3.27 mmol) in acetonitrile (5 mL) was added propargyl alcohol (73 mg, 1.31 mmol) and CuI (15 mg, 0.0763 mmol) while stirring under N_2 . After the addition was complete, the reaction mixture was refluxed for 16 h. At this time, TLC indicated that the starting material was consumed. Solvents were removed *in vacuo* and the residue partitioned between water and chloroform. The phases were separated and chloroform was concentrated

in vacuo. The resulting oil was purified by column chromatography on silica gel (50% EtOAc-hexanes) affording a yellow solid (160 mg, 71%) that was pure by NMR. The product was further purified by recrystallizing from ether-DCM to produce white crystals: mp 87–88 °C. ¹H NMR (400 MHz, CDCl₃) δ 9.13 (s, 1H), 8.86 (s, 1H), 8.33 (s, 1H), 4.53 (d, 2H, *J* = 6 Hz), 4.42 (q, 2H, *J* = 7.2 Hz), 1.41 (t, 3H, *J* = 7.2 Hz). ¹³C NMR (101 MHz, CDCl₃) δ 164.5, 155.3, 149.4, 140.0, 126.0, 120.0, 92.5, 81.0, 61.8, 51.2, 14.2. Anal. (C₁₁H₁₁NO₃) C, H, N. HRMS calcd for C₁₁H₁₁NO₃: 228.0637 (M+Na). Found *m/z*: 228.0634 (M+Na).

5-(3-Hydroxypropyl)nicotinic acid ethyl ester (14)

Compound **13** (270 mg, 1.32 mmol) was dissolved in methanol (8 mL) and transferred to a Pyrex hydrogenation flask and cooled in dry ice-acetone for 5 min. Pd/C (54 mg, 20 % w/w) was added and the flask was attached to a PARR hydrogenator and shaken for 10 h at 30 PSI of H₂. Upon completion, the mixture was filtered through Celite and solvents were removed *in vacuo*. The resulting residue was partitioned between water and chloroform. The chloroform was concentrated under reduced pressure and was purified by column chromatography on silica gel (5% methanol-DCM) affording a yellow oil (245 mg, 89%): TLC R_f 0.37 in 5% methanol-DCM. ¹H NMR (400 MHz, CDCl₃) δ 9.03 (s, 1H), 8.61 (s, 1H), 8.17 (s, 1H), 4.41 (q, 2H, *J* = 7.2 Hz), 3.71 (t, 2H, *J* = 6.4 Hz), 3.62 (s, 1H), 2.83 (t, 2H, *J* = 7.6 Hz), 1.93 (m, 2H), 1.42 (t, 3H, *J* = 7.2 Hz). ¹³C NMR (101 MHz, CDCl₃) δ 165.4, 153.3, 148.2, 137.4, 137.0, 126.1, 61.5, 61.1, 33.7, 29.0, 14.4. HRMS calcd for C₁₁H₁₅NO₃: 232.0950 (M+Na). Found *m/z*: 232.0946 (M+Na).

5-(3-Hydroxypropyl)nicotinic acid (15)

Compound **14** (156 mg, 0.746 mmol) was dissolved in methanol (2 mL) and 4 N NaOH (1mL) and stirred at 37 °C for 4 h. After this time the solvent was evaporated *in vacuo*, the residue dissolved in water and the alkali neutralized using 1 N HCl. After the water was removed by lyophilization, the residue was applied to a combiflash C18 cartridge (15.5 g) and eluted isocratically with water. The mixture was concentrated *in vacuo* and applied to a 35 mL (bed volume) column of Amberchrom resin (CG71M, Dow Chemical Company, product #10235577; Copenhagen, Denmark), which sufficiently desalted the mixture (isocratic, water). Upon drying, a yellowish-white solid was obtained (129 mg, 96 %): mp 216–218 °C. TLC R_f 0.16 in DCM-methanol-acetic acid (90:9:1), TLC R_f 0.69 in butanol-acetic acid-water (5:2:3). ¹H NMR (400 MHz, CD₃OD) δ 8.90 (s, 1H), 8.42 (s, 1H), 8.19 (s, 1H), 3.59 (t, 2H, *J* = 6.4 Hz), 2.77 (t, 2H, *J* = 8 Hz), 1.87 (m, 2H). ¹³C NMR (150 MHz, CD₃OD) δ 173.0, 151.2, 148.9, 139.0, 138.7, 135.0, 61.9, 35.0, 30.1. HRMS calcd for C₉H₁₁NO₃: 182.0817 (M+H). Found *m/z*: 182.0821 (M+H).

5-[3-(*p*-Methylbenzenesulfonyl)propyl]nicotinic acid ethyl ester (16)

Compound **14** (107 mg, 0.51 mmol) was dissolved in dry DCM (5 mL). To this solution was added *p*-methylbenzenesulfonyl chloride (292 mg, 1.5 mmol) and TEA (114 mg, 1.12 mmol). The mixture was refluxed for 16 h. Upon TLC analysis in 50% EtOAc-hexanes, starting material was still present. Additional *p*-toluenesulfonyl chloride (50 mg, 0.26 mmol) and TEA (15 mg, 0.15 mmol) were added to the mixture and allowed to stir at reflux for an

additional 12 h. This pushed the reaction to completion and the product was isolated by removal of solvents by distillation and the residue partitioned between saturated sodium bicarbonate (50 mL) and DCM (50 mL). The organic layer was further washed with brine (3×). The phases were separated and the DCM was evaporated *in vacuo*. The crude material was purified by chromatography on silica gel in 50% EtOAc-hexanes to obtain a colorless oil (94 mg, 51%): TLC R_f 0.27 50% EtOAc-hexanes. ^1H NMR (400 MHz, CDCl_3) δ 9.06 (s, 1H), 8.54 (s, 1H), 8.07 (s, 1H), 7.79 (d, 2H), 7.35 (d, 2H), 4.41 (q, 2H, $J = 7.2$ Hz), 4.06 (t, 2H, $J = 6$ Hz), 2.75 (t, 2H, $J = 8$ Hz), 2.46 (s, 3H), 2.00 (m, 2H), 1.42 (t, 3H, $J = 7.2$ Hz). ^{13}C NMR (101 MHz, CDCl_3) δ 165.4, 153.6, 149.0, 145.2, 136.8, 135.8, 133.0, 130.11, 129.92, 128.0, 126.3, 125.3, 69.2, 61.7, 30.2, 28.7, 21.8, 14.4.

5-(3-Azidopropyl)nicotinic acid ethyl ester (17)

To a solution of **16** (47 mg, 0.13 mmol) in dry DMF (2 mL) was added sodium azide (17 mg, 0.26 mmol). The reaction mixture was stirred and heated for 18 h at 60 °C. Upon completion, solvents were distilled under reduced pressure and the residue purified by column chromatography on silica gel in 50% EtOAc-hexanes yielding a yellow oil (22 mg, 73%): TLC R_f 0.49 in 50% EtOAc-hexanes; R_f 0.77 in DCM/methanol- acetic acid (95:4:1). ^1H NMR (400 MHz, CDCl_3) δ 9.08 (s, 1H), 8.62 (s, 1H), 8.13 (s, 1H), 4.42 (q, 2H, $J = 7.2$ Hz), 3.35 (t, 2H, $J = 6.4$ Hz), 2.79 (t, 2H, $J = 8$ Hz), 1.95 (m, 2H), 1.42 (t, 3H, $J = 7.2$ Hz). ^{13}C NMR (101 MHz, CDCl_3) δ 165.4, 153.5, 148.8, 136.7, 136.1, 126.1, 61.5, 50.4, 30.05, 29.69, 14.3. HRMS calcd for $\text{C}_{11}\text{H}_{14}\text{N}_4\text{O}_2$: 257.1014 (M+Na). Found m/z : 257.1011 (M+Na).

5-(3-Azidopropyl)nicotinic acid (18)

Compound **17** (50 mg, 0.21 mmol) was dissolved in methanol (1 mL) and 4 N NaOH (0.5 mL) and stirred for 3 h at 37 °C. Upon completion, solvents were removed *in vacuo* and the residue was neutralized with 1 N HCl. The sample was concentrated to a volume of 0.5 mL then applied to a C_{18} cartridge (15.5 g) which was then attached to a Combiflash Rf system. The product eluted into the 2nd CV of an isocratic water system. Water was removed by lyophilization to yield a white solid (39 mg, 91%): mp 77–78 °C. TLC R_f 0.40 in DCM-methanol-acetic acid (95:4:1); R_f 0.81 in butanol-acetic acid-water (5:2:3). ^1H NMR (400 MHz, CD_3OD) δ 8.97 (s, 1H), 8.62 (s, 1H), 8.28 (s, 1H), 3.37 (t, 2H $J = 6.4$ Hz), 2.83 (t, 2H, $J = 7.6$ Hz), 1.94 (m, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 168.0, 153.8, 149.1, 139.1, 128.7, 51.8, 31.3, 30.7. HRMS calcd for $\text{C}_9\text{H}_{10}\text{N}_4\text{O}_2$: 207.0882 (M+H). Found m/z : 207.0881 (M+H).

General procedure for the base exchange reaction

Substituted nicotinic acid (10–20 equiv.) was dissolved in water (6 mL) and stirred for 30 min at 37 °C. After this time period, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 min. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH, if necessary. This procedure was repeated until the pH has stabilized. After the pH has stabilized, NADP was added and stirred for 5 min at 37 °C. Next, *Aplysia californica* ADP-ribosyl cyclase (180 μL , 36 μg) was added and the reaction was stirred at 37 °C for 2 h. Upon time completion, HPLC confirmed consumption of starting material (**HPLC Method**

1: NADP $t = 8.96$ min, 13 mM TFA). The sample was then injected onto the preparative HPLC column and the product was purified by anion-exchange chromatography according to **Method 3**. After the product was collected, the combined fractions were added to a 125 mL separatory funnel and extracted 3 $\times$ with DCM (35 mL portions) to remove TFA. The phases were separated and the aqueous layer was then distilled *in vacuo* at 25 mbar for 10 min to remove any residual DCM and TFA. The pH of this resulting solution was adjusted between 7.2- 7.4 with 1 M NaOH. The aqueous solution was allowed to freeze at -80°C then lyophilized. This substance was then dissolved in water (2 mL) and applied to 3 mL column of DEAE cellulose according to **Method 6**. The DEAE cellulose chromatography was developed by applying a 0- 500 mM NH_4HCO_3 gradient and the product eluted into ca. 225 mM NH_4HCO_3 . The product showed UV absorbance at 254 nm; Fractions absorbing at this wavelength were collected and combined. The combined fractions were then lyophilized until a foamy white residue appeared. The sample was redissolved in water (2 mL) and lyophilized. This process was repeated twice more affording pure substituted NAADP derivative.

Preparation of NAADP analogs

NAADP (1)

Nicotinic acid (40 mg, 0.325 mmol) was suspended in water (6 mL) and stirred for 30 min at 37°C . After this time, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 min. The pH was measured again and readjusted to pH = 4 with 0.5 N NaOH, if necessary. This procedure was repeated until the pH has stabilized. After the pH has stabilized, NADP (20 mg, 0.024 mmol) was added and stirred for 5 min at 37°C . Next, *Aplysia* ADP-ribosyl cyclase (180 μL , 36 μg) was added and the reaction was stirred at 37°C for 2 h. After 2 h, analysis by HPLC confirmed consumption of starting material (NADP). The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water-TFA gradients according to **HPLC Method 3**. NAADP eluted between 22.85 and 28.40 min (27–36%). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 min to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2–7.4 with 1 M NaOH. The aqueous solution was frozen then lyophilized producing a white, amorphous solid. The sample was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose according to **Method 6**. The product **1** eluted into 225 mM NH_4HCO_3 . The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved in water (2 mL) and again lyophilized, affording pure NAADP as a white powder: ^1H NMR (400 MHz, D_2O) δ 9.12 (s, 1H), 9.01 (s, 1H), 8.75 (s, 1H), 8.44 (s, 1H), 8.14 (s, 1H), 8.07 (s, 1H), 6.18 (s, 1H), 6.04 (s, 1H), 5.12 (s, 1H), 4.64 (s, 1H), 4.5 (s, 1H), 4.41 (s, 3H), 4.32- 4.21 (m, 4H). MALDI-TOF MS calcd for $\text{C}_{21}\text{H}_{28}\text{N}_6\text{O}_{18}\text{P}_3$ $+$: 745.067. Found m/z : 744.897 (M^+). **HPLC Method 1**: $t = 12.80$, 23 mM TFA. **Method 7**: Product eluted into 300 mM NH_4HCO_3 .

8-N₃-NAD (22)

NMN (15 mg, 0.045 mmol) and **21** (63 mg, 0.13 mmol) were dissolved in a mixed solvent system of H₂O (1 mL) and pyridine (5 mL). Diisopropylcarbodiimide (DIC; 0.23 mL × 5) was added in five portions over 5 d. The mixture was stirred at rt. 24 h after the last addition of DIC, the mixture was poured into H₂O (25 mL) and the mixture was stirred for 2 h. Precipitates were filtered off and the filtrate was lyophilized. The solid residue was dissolved in H₂O (20 mL) and was injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in H₂O-TFA gradients according to **HPLC Method 5**. **22** eluted between 15.76 and 17.23 min (2% 100 mM TFA). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 min to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2–7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in H₂O (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **22** eluted into 50 mM NH₄HCO₃. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure **22** as a cream-colored powder (3.6 mg, 11%): ¹H NMR (400 MHz, D₂O) δ 9.11 (s, 1H), 8.91 (d, 1H, *J* = 6.4 Hz), 8.63 (d, 1H, *J* = 8 Hz), 8.00 (t, 1H, *J* = 8 Hz), 5.91 (d, 1H, *J* = 4.4 Hz), 5.67 (d, 1H, *J* = 5.2 Hz), 4.80–4.08 (m, 10H); ³¹P NMR (162 MHz, D₂O) δ –10.70. **HPLC Method 2**: *t* = 8.69 min, 2 mM TFA.

8-N₃-NADP (23)

8-N₃-NAD (**22**) was converted to 8-N₃-NADP (**23**) enzymatically using NAD kinase. 8-N₃-NAD (**22**) (13.7 mg, 19.5 μmol) was dissolved in 14 mL of 20 mM HEPES, pH 7.3, 3 mM ATP, 5 mM MgCl₂, 2.5 mM creatine phosphate and 0.15 mg/mL creatine kinase. The reaction was initiated by addition of 20 μL of 1 mg/mL human NAD kinase (Axxora) and was allowed to proceed at rt in the dark. After 1 week the reaction had proceeded to 69% completion. The resulting 8-N₃-NADP was purified by chromatography on a 1.5 × 15 cm AG MP-1 column using an HPLC system consisting of a Beckman 128 Solvent Module and a 166 UV detector. The 8-N₃-NADP was eluted at a flow rate of 3 mL/min with a concave upward gradient of TFA from 1.5 to 150 mM over 50 min. 8-N₃-NAD eluted from 15 to 19 min and 8-N₃-NADP from 31 to 35 min. Three mL of the reaction was injected each time and the 8-N₃-NADP peak was pooled from each run. The TFA was extracted from the purified 8-N₃-NADP by treating the pooled fractions (40 mL) with 10 mL of a 3:1 mixture of 1,1,2-trichlorotrifluoroethane/tri-*N*-octylamine.⁵⁷ The resulting aqueous layer was dried in a Savant Speed-Vac concentrator as 10 mL aliquots. A total of 6.4 mg (4.3 μmol) of pure **23** as the octylamine salt was isolated representing a 22 % yield. The 8-N₃-NAD peak was also collected and processed in a similar manner and the NAD kinase reaction repeated as described above to isolate more 8-N₃-NADP: ¹H NMR (600 MHz, D₂O) δ 9.34 (s, 1H), 9.18 (d, 1H, *J* = 6.3 Hz), 8.87 (d, 1H, *J* = 6.7 Hz), 8.23 (m, 2H), 6.09 (d, 1H, *J* = 5.52 Hz), 6.02 (d, 1H, *J* = 5.16 Hz), 5.34 (m, 1H), 4.66 (t, 1H, *J* = 5.22 Hz), 4.54 (m, 1H), 4.48 (t, 1H, *J* = 5.22 Hz), 4.43 (m, 1H), 4.35 (m, 1H), 4.29–4.19 (m, 4H). ³¹P NMR (162 MHz, D₂O) δ 0.61, –10.78. HRMS calcd for C₂₁H₂₈N₁₀O₁₇P₃⁺. 785.084. Found *m/z*: 785.142 (M⁺).

8-Br-NADP (24)

To a stirred solution of NADP (100 mg, 0.12 mmol) in 0.5 M sodium acetate (1.5 mL, pH = 4.75) was added Br₂ (0.2 mL, 4 mmol). The reaction was stirred vigorously using a magnetic stirrer for 3 h while wrapped in aluminum foil to protect from light. Upon completion, the mixture was diluted with water (8.5 mL) then DCM (20 mL) was added. The reaction mixture was transferred to a separatory funnel and the organic layer was discarded. The aqueous layer was further extracted 5 times with DCM (20 mL). The pH was adjusted to 7 with 4 M NaOH and was concentrated *in vacuo* until the total volume was ca. 10 mL. The crude product was purified by anion exchange chromatography according to **HPLC Method 4** in 5 mL portions. 8-Br-NADP eluted between 29.44 and 37.39 min (11–15 mM TFA). Fractions exhibiting UV absorption at 254 nm were combined and extracted 3 times with DCM (10 mL). The aqueous layer was distilled *in vacuo* at 25 mbar for 10 min to remove residual DCM and TFA. The resulting solution was neutralized with 0.5 M NaOH then lyophilized. The amorphous solid was dissolved in water (2 mL) and applied to 3 mL column of DEAE cellulose according to **Method 6**. The product **30** eluted into 175 mM NH₄HCO₃. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 8-Br-NADP as a yellowish-white solid (56 mg, 51%): ¹H NMR (400 MHz, D₂O) δ 9.37 (s, 1H), 9.18 (s, 1H), 8.94 (d, 1H, *J* = 7.6 Hz), 8.31 (s, 1H), 8.20 (s, 1H), 6.21 (s, 1H), 6.11 (s, 1H), 5.53 (s, 1H), 4.5–4.22 (9H). ³¹P NMR (162 MHz, D₂O) 2.09, –10.79. MALDI-TOF MS calcd for C₂₁H₂₇BrN₆O₁₈P₃⁺: 821.994. Found *m/z*: 821.992 (M⁺). **HPLC Method 2**: *t* = 22.72 min, 7 mM TFA.

5-(Acetamido)-NAADP (25)

5-(Acetamido)nicotinic acid (**2c**) (35 mg, 0.194 mmol) was suspended in water (6 mL) and stirred for 30 min at 37 °C. After this time, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 min. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH, if necessary. This procedure was repeated until the pH has stabilized. After the pH has stabilized, NADP (15 mg, 0.018 mmol) was added and stirred for 5 min at 37 °C. Next, *Aplysia* ADP-ribosyl cyclase (180 μL, 36 μg) was added and the reaction was stirred at 37 °C for 4 h. At the end of 4 h, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water-TFA gradients according to **HPLC Method 3**. 5-(Acetamido)-NAADP eluted between 24.12 and 27.91 min (31–37 mM TFA). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 min to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2–7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **25** eluted into 225 mM NH₄HCO₃. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 5-(acetamido)-NAADP as a white powder (7.45 mg, 47%): ¹H NMR (400 MHz, D₂O) δ 9.24 (s, 1H), 9.16 (s, 1H), 8.88 (s,

1H), 8.47 (s, 1H), 8.36 (s, 1H), 6.18 (s, 1H), 6.12 (s, 1H), 5.06 (s, 1H), 4.59 (s, 1H), 4.52 (s, 1H), 4.42–4.23 (7H), 2.18 (s, 3H). ³¹P NMR (162 MHz, D₂O) δ 0.35, - 10.56. **HPLC Method 1:** t = 13.68 min, 25 mM TFA. **Method 7:** 340 mM NH₄HCO₃.

5-Hydroxy-NAADP (26)

5-Hydroxynicotinic acid (**2a**) (35 mg, 0.252 mmol) was suspended in water (6 mL) and stirred for 30 min at 37 °C. After this time, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 min. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH. This procedure was repeated until the pH has stabilized. After the pH has stabilized, NADP (15 mg, 0.018 mmol) was added and stirred for 5 min at 37 °C. Next, *Aplysia* ADP-riboseyl cyclase (180 µL, 36 µg) was added and the reaction was stirred at 37 °C for 2 h. At the end of 2 h, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water-TFA gradients according to **HPLC Method 3**. 5-Hydroxy-NAADP eluted between 25.60 and 29.84 min (32–38 mM TFA). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 min to remove residual DCM and TFA. The pH of this solution was adjusted to between 7.2–7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **26** eluted into 240 mM NH₄HCO₃. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 5-hydroxy- NAADP as a white powder (9.55 mg, 63%): ¹H NMR (400 MHz, D₂O) δ 8.43–8.39 (d, 3H), 8.13 (s, 1H), 7.96 (s, 1H), 6.12 (d, 1H, *J* = 4.8 Hz), 5.82 (s, 1H, *J* = 4.8 Hz), 5.01 (m, 1H), 4.58 (t, 1H), 4.39 (s, 1H), 4.34–4.15 (6H). ³¹P NMR (162 MHz, D₂O) δ 0.91, -10.8. MALDI-TOF MS calcd for C₂₁H₂₈N₆O₁₉P₃⁺: 761.062. Found *m/z*: 761.153 (M⁺). **HPLC Method 1:** t = 14.13 min, 26 mM TFA. **Method 7:** 350 mM NH₄HCO₃.

5-(3-Acetamidopropyl)-NAADP (27)

5-(3-Acetamidopropyl)nicotinic acid (**6**) (37.5 mg, 0.169 mmol) was suspended in water (6 mL) and stirred for 30 min at 37 °C. The pH initially was 8.4 and was adjusted to 4 with 1 N HCl and stirred for 30 min. The pH was measured again and readjusted to pH = 4 with 1 N HCl. After this time, the pH stabilized. NADP (15 mg, 0.018 mmol) was added and stirred for 5 min at 37 °C. Next, *Aplysia* ADP-riboseyl cyclase (180 µL, 36 µg) was added and the reaction was stirred at 37 °C for 5 h. At the end of 5 h, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water-TFA gradients according to **HPLC Method 3**. 5-(3-Acetamidoaminopropyl)-NAADP eluted between 21.87 and 26.24 min (26–33 mM TFA). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 min to remove residual DCM and TFA. The pH of the resulting solution was then

adjusted to between 7.2–7.4 with 1M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **27** eluted into 225 mM NH_4HCO_3 . The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 5-(3-acetamidopropyl)-NAADP as an off-white powder (9.7 mg, 58 %): ^1H NMR (400 MHz, D_2O) δ 9.28 (s, 1H), 9.12 (s, 1H), 8.89 (s, 1H), 8.58 (s, 1H), 8.40 (s, 1H), 6.26 (d, 1H, $J = 4.8$ Hz), 6.11 (d, 1H, $J = 4.8$ Hz), 5.09 (m, 2H), 4.63–4.23 (9H), 3.18 (t, 2H, $J = 6.4$ Hz), 2.97 (t, 2H, $J = 7.6$ Hz), 1.93 (s, 5H). ^{31}P NMR (162 MHz, D_2O) δ 0.39, –10.68. MALDI-TOF MS calcd for $\text{C}_{26}\text{H}_{37}\text{N}_7\text{O}_{19}\text{P}_3^+$: 844.136. Found m/z : 844.217 (M⁺). **HPLC Method 1**: $t = 12.45$ min, 22 mM TFA.

5-(3-*t*-Boc-Aminopropyl)-NAADP (28)

5-(3-*t*-Boc-Aminopropyl)nicotinic acid (**11**) (35 mg, 0.125 mmol) was suspended in water (5 mL) and DMSO (1 mL) and stirred for 30 min at 37 °C. After this time, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 min. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH, if necessary. This procedure was repeated until the pH has stabilized. After the pH has stabilized, NADP (15 mg, 0.018 mmol) was added and stirred for 5 min at 37 °C. Next, *Aplysia* ADP-ribosyl cyclase (180 μL , 36 μg) was added and the reaction was stirred at 37 °C for 6 h. At the end of 6 h, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water-TFA gradients according to **HPLC Method 3**. 5-(3-*t*-Boc-Aminopropyl)-NAADP eluted between 22.57 and 27.75 min (27–34 mM TFA). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 min to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2–7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **28** eluted into 215 mM NH_4HCO_3 . The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 5-(3-*t*-Bocaminopropyl)-NAADP as a white solid (7.6, 42%): ^1H NMR (400 MHz, D_2O) δ 8.96 (s, 1H), 8.79 (s, 1H), 8.62 (s, 1H), 8.42 (s, 1H), 8.09 (s, 1H), 6.11 (s, 1H), 5.96 (s, 1H), 4.60–4.10 (10H), 3.06 (s, 2H), 2.79 (s, 2H), 1.79 (s, 2H), 1.34 (s, 9H). ^{31}P NMR (162 MHz, D_2O) δ 0.44, –10.39. MALDI-TOF MS calcd for $\text{C}_{29}\text{H}_{43}\text{N}_7\text{O}_{20}\text{P}_3^+$: 902.178. Found m/z : 902.278 (M⁺). **HPLC Method 1**: $t = 12.75$ min, 23 mM TFA.

5-(3-Aminopropyl)-NAADP (29)

5-(3-Aminopropyl)nicotinic acid (**12**) (35 mg, 0.194 mmol) was dissolved in water (6 mL) and stirred for 30 min at 37 °C. After this time, the pH was adjusted to 4 with 1 M HCl and stirred for 30 min. The pH was measured again and readjusted to pH = 4 with 1 M HCl. This procedure was repeated until the pH has stabilized. After the pH has stabilized, NADP (15 mg, 0.018 mmol) was added and stirred for 5 min at 37 °C. Next, *Aplysia* ADP-ribosyl

cyclase (180 μ L, 36 μ g) was added and the reaction was stirred at 37 °C for 6 h. At the end of 6 h, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water-TFA gradients according to **HPLC Method 3**. 5-(3-Aminopropyl)-NAADP eluted between 12.83 and 15.44 min (12–16 mM TFA). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 min to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2–7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **29** eluted into 140 mM NH_4HCO_3 . The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 5-(3-aminopropyl)-NAADP as a white powder (10.5 mg, 66%): ^1H NMR (400 MHz, D_2O) δ 8.94 (s, 1H), 8.80 (s, 1H), 8.60 (s, 1H), 8.34 (s, 1H), 8.06 (s, 1H), 6.06 (d, 1H, J = 4.8 Hz), 5.96 (d, 1H, J = 4 Hz), 4.89 (partially buried in HDO peak, 1H), 4.54 (m, 1H), 4.44 (s, 1H), 4.32–4.13 (6H), 3.02 (t, 2H, J = 6.8 Hz), 2.84 (t, 2H, J = 7.6 Hz), 1.97 (m, 2H). ^{31}P NMR (162 MHz, D_2O) δ 4.22, –10.82. MALDI-TOF MS calcd for $\text{C}_{24}\text{H}_{35}\text{N}_7\text{O}_{18}\text{P}_3^+$: 802.125. Found m/z 802.182 (M+). **HPLC Method 1**: t = 6.93 min, 11 mM aq TFA.

5-(3-Hydroxypropyl)-NAADP (30)

5-(3-Hydroxypropyl)nicotinic acid (**15**) (35 mg, 0.193 mmol) was suspended in water (6 mL) and stirred for 30 min at 37 °C. After this time, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 min. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH, if necessary. This procedure was repeated until the pH has stabilized. After the pH has stabilized, NADP (15 mg, 0.018 mmol) was added and stirred for 5 min at 37 °C. Next, *Aplysia* ADP-ribosyl cyclase (180 μ L, 36 μ g) was added and the reaction was stirred at 37 °C for 4 h. At the end of 4 h, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water-TFA gradients according to **HPLC Method 3**. 5-(3-Hydroxypropyl)-NAADP eluted between 21.73 and 26.53 min (26–34 mM aqTFA). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 min to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2–7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product (**30**) eluted into 225 mM NH_4HCO_3 . The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 5-(3-hydroxypropyl)-NAADP as a white powder (9.5 mg, 59%): ^1H NMR (400 MHz, D_2O) δ 8.96 (s, 1H), 8.82 (s, 1H), 8.65 (s, 1H), 8.42 (s, 1H), 8.14 (s, 1H), 6.13 (d, 1H, J = 5.6 Hz), 5.96 (d, 1H, J = 4.4 Hz), 5.03 (m, 2H), 4.6 (m, 1H), 4.45 (s, 1H), 4.38–4.18 (m, 6H), 3.59 (t, 2H, J = 6.4 Hz), 2.86 (t, 2H, J = 8.4 Hz), 1.86 (m, 2H). ^{31}P NMR (162 MHz, D_2O) δ 0.55,

–10.64). MALDI-TOF MS calcd for $C_{24}H_{34}N_6O_{19}P_3^+$: 803.109. Found m/z 803.214 (M+).

HPLC Method 1: $t = 12.70$ min, 23 mM aqTFA. **Method 7:** 310 mM NH_4HCO_3 .

5-(3-Azidopropyl)-NAADP (31)

5-(3-azidopropyl)nicotinic acid (**18**) (35 mg, 0.170 mmol) was suspended in water (5 mL) and DMSO (1 mL) and stirred for 30 min at 37 °C. After this time, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 min. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH, if necessary. This procedure is repeated until the pH has stabilized. After the pH has stabilized, NADP (15 mg, 0.018 mmol) was added and stirred for 5 min at 37 °C. Next, *Aplysia* ADP-ribosyl cyclase (120 μ L, 12 μ g) was added and the reaction was stirred at 37 °C for 5 h. At the end of 5 h, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water-TFA gradients according to **HPLC Method 3**. 5-(3-Azidopropyl)-NAADP eluted between 23.46 and 27.03 min (30–35 mM TFA). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 min to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2–7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **31** eluted into 225 mM NH_4HCO_3 . The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 5-(3-azidopropyl)-NAADP as a white powder (8.5 mg, 52%): 1H NMR (400 MHz, D_2O) δ 9.30 (s, 1H), 9.12 (s, 1H), 8.91 (s, 1H), 8.57 (s, 1H), 8.38 (s, 1H), 6.25 (d, 1H, $J = 5.6$ Hz), 6.10 (d, 1H, $J = 5.2$ Hz), 5.08 (s, 1H), 4.62 (s, 1H), 4.56 (s, 1H), 4.53–4.23 (m, 7H), 3.38 (t, 2H, $J = 6.4$ Hz), 3.02 (t, 2H, $J = 7.2$ Hz), 1.98 (m, 2H). ^{31}P NMR (162 MHz, D_2O) δ 0.39, –10.40. MALDI-TOF MS calcd for $C_{24}H_{33}N_9O_{18}P_3^+$: 828.116. Found m/z : 828.214 (M+). **HPLC Method 1:** $t = 13.17$ min, 24 mM TFA.

5-Thiomethyl-NAADP (32)

5-Thiomethylnicotinic acid (**2d**) (35 mg, 0.207 mmol) was suspended in water (5 mL) and DMSO (1 mL) and stirred for 30 min at 37 °C. After this time, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 min. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH, if necessary. This procedure was repeated until the pH has stabilized. After the pH has stabilized, NADP (15 mg, 0.018 mmol) was added and stirred for 5 min at 37 °C. Next, *Aplysia* ADP-ribosyl cyclase (120 μ L, 24 μ g) was added and the reaction was stirred at 37 °C for 2 h. At the end of 2 h, analysis by HPLC confirmed that the starting material (NADP) had been consumed. The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water-TFA gradients according to **HPLC Method 3**. 5-Thiomethyl-NAADP eluted between 23.60 and 27.89 min (29–35 mM TFA). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 min to remove residual DCM

and TFA. The pH of the resulting solution was adjusted to between 7.2–7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **32** eluted into 225 mM NH_4HCO_3 . The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 5-thiomethoxy-NAADP as a white powder (8 mg, 51%): ^1H NMR (400 MHz, D_2O) δ 9.08 (s, 1H), 8.88 (s, 1H), 8.72 (s, 1H), 8.57 (s, 1H), 8.40 (s, 1H), 6.25 (s, 1H), 6.07 (s, 1H), 5.08 (s, 1H), 4.62–4.20 (9H), 2.65 (s, 3H). ^{31}P NMR (162 MHz, D_2O) δ 0.31, –10.72. MALDI-TOF MS calcd for $\text{C}_{22}\text{H}_{30}\text{N}_6\text{O}_{18}\text{P}_3\text{S}^+$: 791.055. Found m/z : 791.139 (M+). **HPLC Method 1**: $t = 13.44$ min, 25 mM TFA.

8-Bromo-NAADP (33)

Nicotinic acid (35 mg, 0.207 mmol) was dissolved in water (5 mL) and DMSO (1 mL) and stirred for 30 min at 37 °C. After this time, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 min. The pH was measured again and readjusted to pH 4 with 0.5 M NaOH, if necessary. This procedure was repeated until the pH has stabilized. After the pH has stabilized, 8-Br-NADP (15 mg, 0.018 mmol) was added and stirred for 5 min at 37 °C. Next, *Aplysia* ADP-ribosyl cyclase (180 μL , 36 μg) was added and the reaction was stirred at 37 °C for 2 h. At the end of 2 h, analysis by HPLC confirmed that the starting material **24** had been consumed. The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in water-TFA gradients according to **HPLC Method 3**. 8-Br-NAADP eluted between 23.00 and 27.97 min (28–35 mM TFA). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 min to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2–7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **33** eluted into 220 mM NH_4HCO_3 . The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure 8-Br-NAADP as a yellowish-white powder (8 mg, 51%): ^1H NMR (400 MHz, D_2O) δ 9.22 (s, 1H), 9.01 (d, 1H, $J = 6.4$ Hz), 8.89 (d, 1H, $J = 8$ Hz), 8.25 (s, 1H), 8.18 (m, 1H), 6.21 (d, 1H, $J = 4$ Hz), 6.04 (d, 1H, $J = 5.2$ Hz), 5.49 (m, 1H), 4.91 (m, 1H), 4.49–4.31 (8H). ^{31}P NMR (162 MHz, D_2O) δ 5.17, –9.91. MALDI-TOF MS calcd for $\text{C}_{21}\text{H}_{27}\text{BrN}_6\text{O}_{18}\text{P}_3^+$: 822.978. Found m/z : 823.208 (M+). **HPLC Method 1**: $t = 13.47$ min, 25 mM TFA.

8-azido-NAADP (34)

2.6 mg (1.74 μmol) of 8- N_3 -NADP (**23**) trioctylammonium salt was dissolved in 2.2 mL water. The solution was adjusted to 100 mM sodium acetate by adding 0.8 mL of 500 mM sodium acetate, pH 4.0. Nicotinic acid (1 mL of 200 mM) was added and the base-exchange reaction was started by adding 5 μL of 200 $\mu\text{g/mL}$ *Aplysia* ADP-ribosyl cyclase and allowed to incubate at room temperature in the dark. The reaction was monitored by analyzing 50 μL of reaction on the analytical chromatography system. The reaction was complete at 6 h as

judged by the disappearance of 8-N₃-NADP. The 8-N₃-NAADP was purified using the same system as described for 8-N₃-NADP (**23**) after adjusting the pH of the reaction to 6 by adding 200 μ L of 2 M Tris-base. 8-N₃-NAADP eluted between 41 and 45 min. The purified 8-N₃-NAADP was processed as described for 8-N₃-NADP (**23**), resulting in the isolation of 1.2 mg (0.65 μ mol; 37 % yield) of **34** isolated as the trioctylammonium salt: ¹H NMR (600 MHz, D₂O) δ 8.99 (s, 1H), 8.93 (d, 1H, *J* = 6.6 Hz), 8.63 (d, 1H, *J* = 8.16 Hz), 8.06 (s, 1H), 7.99 (t, 1H, *J* = 7.02 Hz), 5.93 (d, 1H, *J* = 4.98 Hz), 5.90 (d, 1H, *J* = 5.34 Hz), 5.23 (m, 1H), 4.29-4.12 (m, 9H). ³¹P NMR (162 MHz, D₂O) δ 0.59, -10.76. MALDI-TOF MS calcd for C₂₁H₂₇N₉O₁₈P₃⁺: 786.068. Found *m/z*: 786.068 (M⁺).

8-Bromoadenosyl-5-azido-NAADP (**35**)

5-Azido-nicotinic acid (33 mg, 0.201 mmol) was dissolved in H₂O (5mL) and DMSO (1 mL) and stirred for 30 min at 37 °C. After this time period, the pH was adjusted to 4 with 0.5 M NaOH and stirred for 30 min. The pH was measured again and readjusted to pH = 4 with 0.5 M NaOH, if necessary. This procedure was repeated until the pH has stabilized. After the pH has stabilized, 8-Br-NADP (15 mg, 0.018 mmol) was added and stirred for 5 min at 37 °C. Next, *Aplysia* ADP-ribosyl cyclase (180 μ L, 36 μ g) was added and the reaction was stirred at 37 °C for 5 h. HPLC confirmed that the starting material had been consumed. The sample was then injected onto the preparative HPLC column and the product was purified by anion exchange chromatography in H₂O-TFA gradients according to **HPLC Method 3**. **35** eluted between 31.04 and 35.76 min (30–37 mM TFA). The combined aqueous fractions were added to a 125 mL separatory funnel and extracted 3 times with DCM (35 mL portions). The phases separated and the aqueous layer was distilled *in vacuo* at 25 mbar for 10 min to remove residual DCM and TFA. The pH of the resulting solution was adjusted to between 7.2–7.4 with 1 M NaOH. The aqueous solution was frozen and lyophilized producing a white, amorphous solid. The amorphous solid was dissolved in water (2 mL) and applied to a 3 mL column of DEAE cellulose, and purified according to **Method 6**. The product **35** eluted into 225 mM NH₄HCO₃. The fractions showing UV absorption at 254 nm were combined and lyophilized. The sample was redissolved and again lyophilized, affording pure **35** as a light yellow powder (5 mg, 35%): ¹H NMR (600 MHz, D₂O) δ 8.88 (s, 1H), 8.64 (s, 1H), 8.57 (s, 1H), 8.15 (s, 1H), 6.13 (d, 1H, *J* = 4.4 Hz), 5.89 (d, 1H, *J* = 4.2 Hz), 5.39 (m, 1H), 4.41-4.19 (m, 9H). ³¹P NMR (162 MHz, D₂O) δ 5.00, -10.39. MALDI-TOF MS calcd for C₂₁H₂₆BrN₉O₁₈P₃⁺: 863.979. Found *m/z*: 863.973 (M⁺). **HPLC Method 1**: *t* = 15.04 min, 29 mM TFA. Method 7: 300 mM NH₄HCO₃.

Biological Assays

All compounds tested (**1**, **25–35**) were re-purified by preparative anion exchange chromatography immediately prior to their bio-assay and the purity of the collected fractions verified by analytical anion exchange HPLC (AG MP-1). The purity of the tested compounds was in all cases 95 % (see Supporting Information).

Ca²⁺ Release—Testing of NAADP derivatives for Ca²⁺ release on cell free receptor systems were performed on homogenates (1.25% v/v) prepared from sea urchin eggs (*Strongylocentrotus purpuratus*) diluted with intracellular medium containing 250 mM potassium gluconate buffer (pH 7.2), 0.5 mM ATP, 4 mM creatinine phosphate, creatinine

kinase and 3 μM fluorescent indicator, fluo-3. The dilutions and all experiments were conducted at 17 °C. Fluo-3 is a calcium chelating indicator which is nonfluorescent in absence of Ca^{2+} ions but after binding Ca^{2+} it emits fluorescence. This fluorescence can be measured using a fluorescence plate reader (excitation 490 nm and emission 535 nm, suitable to avoid interference from reduced pyridine nucleotide).⁵⁸

Receptor desensitization—Sub-threshold concentrations of tested analogs were added to a standard Ca^{2+} release assay and incubated for the specified time before the addition of saturating concentrations of NAADP.

Receptor binding—The competitive binding studies were performed according to our previously published procedures.⁵⁸ The binding assays were done in triplicate in 96 well filter plates containing the cell free sea urchin egg homogenate and constant concentration of radioligand [^{32}P]NAADP (0.2 nM). Seven concentrations of each compound were tested to determine their IC_{50} . The competitor and [^{32}P]NAADP were incubated simultaneously with the sea urchin egg homogenate for 90 min at 4 °C. The homogenate was filtered and washed and the radioactivity retained on the filter was determined by liquid scintillation. Non-specific binding was defined as radioactivity not displaced by 1 μM NAADP and represented between 0.05 and 2% of total binding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

cADPR	cyclic adenosine diphosphate ribose
CV	column volume in reference to column chromatography
DEAE	diethylaminoethyl group
DIPEA	diisopropylethylamine
EtOAc	ethyl acetate
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid buffer
IP3	D-myo-inositol trisphosphate
NAADP	nicotinic acid adenine dinucleotide phosphate
PTFE	polytetrafluoroethylene
<i>t</i>-Boc	<i>tert</i> -butyloxycarbonyl protecting group
TEA	triethylamine

TPC two pore channel

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Figure 1.
Enzyme catalyzed base-exchange reaction.

Figure 2. Spectrophotometric titration of 5-hydroxy-NAADP (**26**). Panel A (left) Electronic absorption spectra of **26** determined at varying pH values. Panel B (right) $A_{335\text{nm}}$ versus pH.

Figure 3.

Typical concentration response curves for NAADP (**1**) (Panel A, left) and analog 5-thiomethyl-NAADP (**32**) (Panel B, right). The experiments were performed in sea urchin egg homogenates. The black line represents Ca^{2+} release as measured by the increase in fluo 3 fluorescence (left axis). The blue line corresponds to specific binding in [^{32}P]NAADP competition binding experiments. The red line depicts Ca^{2+} release after desensitization. This data is illustrative of potent agonists to the NAADP receptor.

Figure 4.

Concentration response curves for 5-(3-azidopropyl)-NAADP (**31**). The experiments were performed in sea urchin egg homogenates. The black line represents Ca²⁺ release. The blue line corresponds to specific binding in [³²P]NAADP competition binding experiments. The red line is measuring Ca²⁺ release after desensitization. This data is illustrative of a partial agonist to the NAADP receptor.

Scheme 1. Synthesis of 5-(2-Phenylethyl)-3-pyridinecarboxylic acid (2f)^a

^aReagents and conditions: (a) phenylacetylene, Pd(OAc)₂, CuI, DIPEA, triphenylphosphine, acetonitrile, reflux 16 h, stirring under N₂, 82%; (b) H₂, Pd on C, EtOAc/methanol 10 h, 82%; (c) NaOH, CH₃OH, 18 h ii) HCl, 71%.

Scheme 2. Synthesis of 5-(3-Acetamido)nicotinic Acid (6)^a

^aReagents and conditions: (a) **3**, Pd(PPh₃)₂Cl₂, CuI, DIPEA, CH₃CN, reflux, 18 h, 60%; (b) H₂, Pd/C, CH₃OH, 8 h, 91%; (c) i) NaOH, CH₃OH, 3.5 h ii) HCl, 89%.

Scheme 3. Synthesis of Compounds 11 and 12a

^aReagents and conditions: (a) **7**, Pd(PPh₃)₂Cl₂, CuI, DIPEA, CH₃CN, reflux, 6 h, 61%; (b) H₂, Pd/C, CH₃OH, 6 h, 93%; (c) TFA, CH₃OH, 37 °C, 2 h; (d) NaOH, CH₃OH, 8 h, 89%; (e) i) NaOH, CH₃OH, 37 °C, 4 h ii) HCl, 83%.

Scheme 4. Synthesis of Compound 18a

^aReagents and conditions: (a) Propargyl alcohol, Pd(PPh₃)₂Cl₂, CuI, DIPEA, CH₃CN, reflux, 16 h, 71%; (b) H₂ and Pd/C, CH₃OH, 10 h, 89%; (c) i) NaOH, CH₃OH, 37 °C, 4 h ii) HCl, 96%; (d) *p*-methylbenzenesulfonylchloride, TEA, CH₂Cl₂, reflux 28 h, 51%; (e) NaN₃, DMF, 60 °C, 18 h, 73%; (f) i) NaOH, CH₃OH, 37 °C, 3 h ii) HCl, 91%.

Scheme 5. Synthesis of 8-N₃-NADP (23)^a

^aReagents and Conditions: (a) Anhydrous hydrazine, CH₃CH₂OH, reflux; (b) i) *Iso*-amyl nitrite, 1 N HCl, ii) 1 N NaOH; (c) i) POCl₃, trimethylphosphate ii) 1 N NaOH, pH = 7; (d) Nicotinamide mononucleotide, diisopropylcarbodiimide, pyridine, H₂O; (e) NAD Kinase and ATP.

Scheme 6. Bromination of NADP to Produce 24a

^aReagents and conditions: (a) Br₂, 0.5 N sodium acetate, pH = 4.

Table 1

5-Substituted pyridine-3-carboxylic acids (**2a–f**) obtained and evaluated as potential substrates for the chemoenzymatic synthesis designed to produce novel pyridinium substituted NAADP derivatives.

Structure	Cmpd	Substituent	Base-exchange product detected
<p>2a–f, 6, 11, 12, 15, 18</p>	2a	-OH	yes
	2b	-Br	no
	2c	-NH-COCH ₃	yes
	2d	-SCH ₃	yes
	2f	-CH ₂ -CH ₂ -C ₆ H ₅	no
	6	-CH ₂ -CH ₂ -CH ₂ -NH-COCH ₃	yes
	11	-CH ₂ -CH ₂ -CH ₂ -NH-t-Boc	yes
	12	-CH ₂ -CH ₂ -CH ₂ -NH ₃ ⁺	yes
	15	-CH ₂ -CH ₂ -CH ₂ -OH	yes
	18	-CH ₂ -CH ₂ -CH ₂ -N ₃	yes

Table 2

Mono- and di-substituted NAADP analogs obtained using our chemoenzymatic synthesis

Structure	R ₁	R ₂	#	Name	Yield
	-NHCOCH ₃	-H	25	5-acetamido-NAADP	47%
	-OH	-H	26	5-hydroxy-NAADP	63%
	-CH ₂ CH ₂ CH ₂ -NH-Ac	-H	27	5-(3-acetamidopropyl)-NAADP	58%
	-CH ₂ CH ₂ CH ₂ -NH- <i>t</i> -Boc	-H	28	5-(3- <i>t</i> -Boc-aminopropyl)-NAADP	42%
	-CH ₂ CH ₂ CH ₂ -NH ₃ ⁺	-H	29	5-(3-aminopropyl)-NAADP	66%
	-CH ₂ CH ₂ CH ₂ -OH	-H	30	5-(3-hydroxypropyl)-NAADP	59 %
	-CH ₂ CH ₂ CH ₂ -N ₃	-H	31	5-(3-azidopropyl)-NAADP	52%
	-SCH ₃	-H	32	5-(thiomethyl)-NAADP	51%
	-H	-Br	33	8-bromo-NAADP	60%
	-H	-N ₃	34	8-azido-NAADP	37%
	-N ₃	-Br	35	5-azido-8-bromo-NAADP	35%

Table 3

EC₅₀ values for Ca²⁺ release induced by NAADP (**1**) and NAADP analogs (**25–35**) measured fluorometrically from Ca²⁺ loaded sea urchin egg homogenates.

Compound	EC ₅₀ ± s.e.m., nM (n)	Fold increase in EC ₅₀
NAADP (1)	25.8 ± 5.7 (19)	1
5-hydroxy-NAADP (26)	1932.3 ± 440.1 (3)	74.9
5-acetamido-NAADP (25)	626.2 ± 46.1 (7)	24.3
5-thiomethyl-NAADP (32)	151.3 ± 22.8 (3)	5.9
5-(3-hydroxypropyl)-NAADP (30)	4433.0 ± 578.7 (7)	171.8
5-(3-azidopropyl)-NAADP (31)	30749 ± 5654 (8)	1191.8
5-(3- <i>t</i> -Boc-aminopropyl)-NAADP (28)	160021 ± 54720 (9)	6202.4
5-(3-aminopropyl)-NAADP (29)	>1000000 (3)	>33333
5-(3-acetamidopropyl)-NAADP (27)	41718 ± 7097 (6)	1617
8-bromo-NAADP (33)	709.1 ± 63.6 (3)	27.5
8-azido-NAADP (34)	47.7 ± 1.6 (3)	1.8
5-azido-8-bromo-NAADP (35)	1363000 ± 145600 (3)	52829

Table 4IC₅₀ values determined for competition ligand binding between [³²P]NAADP and NAADP analogs (**25–35**)

Compound (structure #)	IC ₅₀ ± s.e.m., nM (n)	Fold increase in IC ₅₀
NAADP (1)	0.37 ± 0.03 (16)	1
5-hydroxy-NAADP (26)	18.9 ± 4.9 (3)	51
5-acetamido-NAADP (25)	15.0 ± 3.0 (4)	40.5
5-thiomethyl-NAADP (32)	6.8 ± 3.3 (3)	18.4
5-(3-hydroxypropyl)-NAADP (30)	49.0 ± 15.2 (4)	132.4
5-(3-azidopropyl)-NAADP (31)	56.3 ± 12.8 (4)	152.2
5-(3- <i>t</i> -Boc-aminopropyl)-NAADP (28)	3270 ± 1720 (4)	8840
5-(3-aminopropyl)-NAADP (29)	> 1 mM (4)	> 2 × 10 ⁶
5-(3-acetamidopropyl)-NAADP (27)	1800 ± 967 (3)	4878
8-bromo-NAADP (33)	15.7 ± 2.1 (3)	42.4
8-azido-NAADP (34)	0.7 ± 0.2 (4)	1.9
5-azido-8-bromo-NAADP (35)	372 (1)	1005

Table 5

IC₅₀ values determined for desensitization of calcium release induced by 1μM NAADP after a 7 minute preincubation with varying concentrations of NAADP analogs (**25–35**)

Compound (structure #)	IC ₅₀ ± s.e.m., nM (n)	Fold increase in IC ₅₀
NAADP (1)	0.47 ± 0.08 (6)	1
5-hydroxy-NAADP (26)	23.1 ± 7.7 (3)	49.1
5-acetamido-NAADP (25)	21.5 ± 7.9 (3)	45.7
5-thiomethyl-NAADP (32)	4.2 ± 0.6 (3)	8.9
5-(3-hydroxypropyl)-NAADP (30)	122.0 ± 21.8 (3)	259.6
5-(3-azidopropyl)-NAADP (31)	45.6 ± 3.8 (3)	97
5-(3- <i>t</i> -Boc-aminopropyl)-NAADP (28)	942 ± 359 (7)	2004
5-(3-aminopropyl)-NAADP (29)	674000. (1)	1434042.
5-(3-acetamidopropyl)-NAADP (27)	1230.6 ± 174.6 (6)	22618.3
8-bromo-NAADP (33)	15.3 ± 5.4 (3)	32.6
8-azido-NAADP (34)	0.6 ± 0.2 (2)	1.3
5-azido-8-bromo-NAADP (35)	575.5 ± 57.4 (3)	1224.5