PHD

Chemical tools to study NAADP: A novel calcium mobilising intracellular messenger

Brown, Richard Stephen

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Chemical tools to study NAADP, a novel calcium mobilising intracellular messenger.

Richard Stephen Brown
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University of Bath,
Department of Pharmacy and Pharmacology
November 2004

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Date 24/01/2005
Abstract

Nicotinic acid adenine dinucleotide phosphate (NAADP) 3 has recently emerged as the most potent endogenous calcium mobilising, second messenger. NAADP 3 has been shown to be essential in many different cell types, ranging from mediating fertilisation in sea urchin eggs to the release of insulin from pancreatic β-cells. Therefore, a synthetic route was required to enable the formation of NAADP 3 and its analogues to explore its intracellular chemistry.

Our aim was to develop a flexible route to NAADP 3 that allowed modifications at key positions, ultimately leading to a range of biological probes. Achieving selective introduction of the 2'-phosphate onto suitably protected adenosine was key. The 5'-hydroxyl was then phosphorylated to afford the protected adenosine diphosphate precursor. Development of a synthetic route towards β-nicotinamide mononucleotide (β-NMN) 21 started from the tetra-protected ribose 30. Activating the anomeric acetate to allow glycosylation to occur with in-situ protecting group cleavage afforded the mononucleoside precursor, which was selectively phosphorylated at the 5'-hydroxyl the afford β-NMN 21. With the two phosphate precursors synthesised, development of a method towards pyrophosphate formation was undertaken. Reported yields for pyrophosphate formation are generally poor due to apparent purification difficulties, however activation with carbonyl diimidazole and purification on negative ion exchange resin afforded pleasing yields of around 60 %. Global deprotection to afford NADP 7 initially failed due to protecting group incompatibilities; however with protecting group alteration formation of NADP 7 was achieved. Enzymatic base-exchange with Aplysia ADP-ribosyl cyclase [E.C. 3.2.2.5] was achieved to afford the synthesis of NAADP 3, which was proven active by biological assay. Analogues were formed using this scheme and evaluated.
Publications


Acknowledgments

I would like to thank Dr. James Dowden for providing me with the opportunity to undertake this research, and for his continuing energy and enthusiasm throughout the project. I would also like to thank Professor Barry Potter for his support during the course of my PhD.

I would like to express my gratitude to Dr. Christelle Moreau, who worked tirelessly with me on the synthesis and provided me with help and guidance throughout. I would also like to thank Dr. Charles Borissow for helpful discussions and practical guidance on techniques and principles within organic synthesis. Drs. C Moreau, S Bartlett and S Foster are also thanked for constructive criticism and proofreading of this thesis.

For their technical support I would like to thank Dr Steve Black for the NMR spectra, Mr. Chris Cryer for mass spectrometry and Mr. Kevin Smith for his technical support.

For the pharmacological evaluation presented here I would like to thank the group of Professor Antony Galione in the Pharmacology department at the University of Oxford.
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>[α]_D</td>
<td>specific rotation at 589 nm</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>β-NAD</td>
<td>β-nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>β-NADP</td>
<td>β-nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>β-NaMN</td>
<td>β-nicotinamide mononucleotide</td>
</tr>
<tr>
<td>β-NMN</td>
<td>β-nicotinamide mononucleotide</td>
</tr>
<tr>
<td>BSA</td>
<td>N,O-bis(trimethylsilyl) acetamide</td>
</tr>
<tr>
<td>cADPR</td>
<td>cyclic adenosine 5'-diphosphate ribose</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CDI</td>
<td>carbonyl diimidazole</td>
</tr>
<tr>
<td>CICR</td>
<td>calcium induced calcium release</td>
</tr>
<tr>
<td>CSO</td>
<td>(1S)-(+-)(10-camphorsulphonyl) oxaziridine</td>
</tr>
<tr>
<td>d</td>
<td>doublet (spectral)</td>
</tr>
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<td>diacetyl glycerol</td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-diazabicyclo[5.4.0]undec-7-ene</td>
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<td>DCC</td>
<td>N,N'-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>dd</td>
<td>double doublet (spectral)</td>
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<tr>
<td>DMAP</td>
<td>4-dimethylaminopyridine</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine 5'-diphosphate</td>
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<tr>
<td>GPN</td>
<td>glycyl-phenylalanine 2-naphthylamide</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
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<tr>
<td>Ins(1,3,4,5)P_4</td>
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</tr>
<tr>
<td>Ins(1,4,5)P_3R</td>
<td>inositol-(1,4,5)-trisphosphate receptor</td>
</tr>
<tr>
<td>J</td>
<td>coupling constant (in NMR)</td>
</tr>
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<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
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<tr>
<td>m/z</td>
<td>mass to charge ratio (mass spectrometry)</td>
</tr>
<tr>
<td>mCPBA</td>
<td>meta chloroperoxybenzoic acid</td>
</tr>
<tr>
<td>mp</td>
<td>melting point</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NAAD</td>
<td>nicotinic acid adenine dinucleotide</td>
</tr>
<tr>
<td>NAADP</td>
<td>nicotinic acid adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NAADPH</td>
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</tr>
<tr>
<td>NADPH</td>
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</tr>
<tr>
<td>NDC</td>
<td>1-(2,4-dinitrophenyl)-3-carbamoyl-pyridinium chloride</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>PI(3)K</td>
<td>phosphatidylinositol-3-OH kinase</td>
</tr>
<tr>
<td>PLCβ</td>
<td>phospholipase C-β</td>
</tr>
<tr>
<td>PLCγ</td>
<td>phospholipase C-γ</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>PMCA</td>
<td>plasma membrane calcium ATPase</td>
</tr>
<tr>
<td>ppm</td>
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<tr>
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<tr>
<td>R&lt;sub&gt;F&lt;/sub&gt;</td>
<td>retention factor (TLC)</td>
</tr>
<tr>
<td>ROC</td>
<td>receptor-operated channels</td>
</tr>
<tr>
<td>R&lt;sub&gt;T&lt;/sub&gt;</td>
<td>retention time (HPLC)</td>
</tr>
<tr>
<td>RYR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>s</td>
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<tr>
<td>SAR</td>
<td>structure activity relationship</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco endoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>SOC</td>
<td>store-operated channels</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<tr>
<td>t</td>
<td>triplet (spectral)</td>
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<td>TBAF</td>
<td>tetrabutylammonium fluoride</td>
</tr>
<tr>
<td>TEAB</td>
<td>Tetrabutylammonium bicarbonate</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TFAA</td>
<td>trifluoroacetic anhydride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TIPDS</td>
<td>tetraisopropyl disiloxane</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
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<td>tetramethylguanidine</td>
</tr>
<tr>
<td>TMS</td>
<td>trimethylsilyl</td>
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<tr>
<td>TMSCN</td>
<td>trimethylsilyl cyanide</td>
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<td>TMSOTf</td>
<td>trimethylsilyl trifluoromethanesulfonate</td>
</tr>
<tr>
<td>UMP</td>
<td>uridine 5'-monophosphate</td>
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<tr>
<td>VOC</td>
<td>voltage-operated channels</td>
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Introduction

The versatile role of calcium
1.1 Overview of calcium signalling

Calcium is a ubiquitous intracellular messenger that controls a diverse range of cellular functions. Calcium is versatile, playing a pivotal role in cellular processes such as controlling fertilisation, gene transcription, muscle contraction, cell proliferation and secretion of bioactive compounds (Figure 1).\textsuperscript{1-4} These processes occur despite the fact that calcium is highly toxic, causing necrosis and programmed cell death (apoptosis).\textsuperscript{1,3,5} How calcium orchestrates these specific processes at the cellular level is the subject of intense study, but can be rationalised by the ability of the signal to be modulated in terms of speed, amplitude and spatio-temporal patterning.\textsuperscript{3}

![Figure 1: Overview of the effect of insulin release mediated by calcium in response to external stimuli.](image)

Resting cells possess a calcium concentration of 100 nM, however stimulation elevates this level to around 1000 nM.\textsuperscript{2,3} The intracellular concentration is finely controlled by an extensive repertoire of signalling components, which when stimulated, activate the ‘on’ mechanisms, to cause a gradient driven influx of calcium.
into the cytoplasm (1-2 mM extracellular concentration). As a messenger, calcium activates a series of dependent processes before finally, the 'off' mechanism re-establishes the resting state by pumping calcium into stores (Figure 2). The 'on' mechanisms include channels located on the plasma membrane (PM) to regulate the supply of calcium from the extracellular space and channels located on the endoplasmic or sarcoplasmic reticulum (ER/SR) to control the internal movement from selected organelles. Calcium can only travel a short distance within the cytoplasm due to rapid binding to various buffers such as parvalbumin, calbindin-D$_{28K}$ and calretinin allowing tight control over movement around the cytoplasm. Release can therefore either be confined to a specific spatial region (e.g. the secretory pole of an acinar cell) or can be cell wide, propagating a wave that spreads through the entire cytoplasm (e.g. egg fertilisation by sperm). The 'off' mechanism functions to remove calcium from the cytoplasm through either, plasma membrane calcium ATPase (PMCA) pumps and sodium/calcium exchangers to extrude calcium into the extracellular domain or, sarco-endoplasmic reticulum calcium ATPase (SERCA) pumps to internally store calcium in selected organelles. Mitochondria play an important role in this regulation by having a low affinity, yet high capacity, rapid calcium uniporter to significantly reduce the cytosol concentration, while allowing slow release back into the cytosol during the recovery phase. Due to their low sensitivity to calcium, mitochondria function more effectively when closely linked to calcium releasing channels that allow reciprocal interaction with the ER/SR. This diversity enables the huge variability in role and function in the signals recorded within different cell types.
Figure 2: The units of the calcium signalling network to generate the ON signal causing downstream events. The response is terminated by the off signal.

There are three main types of channel responsible for the external influx of calcium into the cell. All produce a local effect due to brief opening times and the action of calcium chelating proteins, but only two of these are well documented.\(^2,^3,^5\) Most is known about voltage-operated channels (VOC's) that respond to transient membrane depolarisation and receptor-operated channels (ROC's), that are often coupled to G-proteins. Downstream calcium release in ROC's is normally in response to external stimuli such as, vasopressin, acetylcholine and ATP. There is also a third class, of uncertain identity, that respond to internal store depletion called store-operated channels (SOC's).\(^3\)

VOC's are found mainly on excitable cell types including neuronal and muscle cells, where activation is a result of depolarisation of the PM. These channels are comprised of five main subunits, but an array of isoforms of these subunits allows many different possibilities that are conventionally characterised by their different gating properties and pharmacology.\(^2\) VOC's increase calcium concentration in the muscle cytosol, either as a result of direct influx through the channel, or downstream activation of a channel embedded in the SR. Neuronal activation comprises many different sub-types of VOC found either at the synapse to trigger exocytosis of neurotransmitters, or along the dendrite to induce gene transcription leading to memory and learning.\(^3\)
ROC's comprise a wide range of structurally diverse channels, which all rely on the activation by external stimuli. ROC's include the N-methyl-D-aspartame receptor, which in neurons, is responsible for early synaptic modifications that result in learning and memory. These channels either directly allow gradient driven influx of calcium, or are coupled to G-proteins, generating the release of small, water soluble second messengers that rapidly diffuse across the cytosol, bind to receptors on the ER/SR, and thus initiate the calcium release.

SOC's respond to depletion of intracellular stores of calcium, however what is unclear is the manner in which this occurs. The ability of the SOC to 'sense' calcium levels has led speculation towards a coupling between calcium entry channels on the PM and release channels on the ER/SR. Since depleting cell calcium pools has been shown to enhance calcium entry, these SOC's may be the most ubiquitous of the PM calcium channels.

It is at these PM bound receptors where the variety of drugs altering calcium release act. They have been used to treat a variety of conditions such as high blood pressure, cardiac dysrhythmias and angina. By working to inhibit calcium influx into cells they promote the relaxation of the blood vessels (e.g. nifedipine), or they reduce the workload of the heart by making it less excitable (e.g. verapamil). Both of these drugs provide very effective treatment, yet for the ubiquity of calcium, this is one very small area of medicine.

The calcium signal can also be derived from internal stores mediated by an array of distinct, messenger-activated channels located on either the ER, or, the SR in muscle cells. Release from these internal stores is initially a result of the messenger, but quickly escalates due to calcium induced calcium release (CICR). There are various channels controlled by this mechanism of which the inositol-1,4,5-trisphosphate receptor (InsP₃R) and ryanodine receptor (RYR) have been extensively studied. This release is initiated by external stimuli binding to cell surface receptors causing the synthesis of second messengers, which diffuse across the cell before binding to their respective receptor on the ER/SR. Two such second messengers are inositol-1,4,5-trisphosphate (Ins(1,4,5)P₃) 1 and cyclic ADP-ribose (cADPR) 2 (Figure 3), which act on the InsP₃R and RYR respectively. Although InsP₃R's require Ins(1,4,5)P₃ 1 to open, their activity is modulated by the concentration of calcium in the neighbouring cytosol and release is enhanced by a modest cytosolic calcium
concentration (0.5–1 μM) via CICR, however large calcium concentrations (>1 μM) inhibit opening. RYR are structurally and functionally similar to InsP3R, however they are activated and inhibited by higher concentrations of calcium (1–10 μM: >10 μM). RYR are located mainly in excitable cells such as muscle or neurons, unlike InsP3R, which are universally expressed throughout mammalian tissues.2

![Figure 3](image)

The apparent wave of calcium that sweeps across the entire cell has been attributed to CICR, such as that observed during oocytes fertilisation. At low levels of stimulation individual Ins(1,4,5)P3R’s or RYR’s open to release calcium characterised as either a ‘blip’ or a ‘quark’. Further stimulation causes clusters of channels to open with the resultant release of either a ‘puff’ or a ‘spark’, which can then develop into a self-propagating wave. Gap junctions allow the calcium waves to bridge the space between separate cells to generate intercellular waves, however the exact mechanism of this is unclear. What is apparent is that this allows many cells to function as one, for example, the wave stimulates lung epithelial cells to beat their cilia in tandem to expel inhaled contaminants.3

Other messengers have been studied, namely the sphingolipids, cyclic AMP (cAMP), cyclic GMP (cGMP) and nicotinic acid adenine dinucleotide diphosphate (NAADP) 3. The properties of the sphingolipid receptors bear no similarities to those of Ins(1,4,5)P3 or RYR and even though they are expressed in many different cell tissues the physiological function of this channel is poorly understood. Upon external stimulation, AMP and GMP are modified by adenyl and guanyl cyclase respectively to form either cAMP or cGMP, which is intimately linked to calcium concentration through its action on channels and pumps. With calcium concentrations initiating both a positive and negative feedback mechanism, depending on which isoform of adenyl cyclase is acted upon, tight regulation of further cAMP/cGMP synthesis can be
achieved. NAADP 3 is emerging as a novel and highly potent mobilising agent of calcium that is only just beginning to be understood. It has been shown to act as a second messenger, but the identity of its receptor is still a topic of huge debate. NAADP 3 will be discussed at far greater depth later in this chapter, however the actions of the sphingolipids, cAMP and cGMP will not be further elaborated.

1.2 Intracellular calcium signalling – Ins(1,4,5)P₃

External stimuli, such as hormones, interact with PM bound, G-protein coupled receptors or tyrosine kinase linked receptors to induce downstream events, mediated by calcium. G-protein coupled receptors allow the activation of a trimeric (α, β and γ) Gq-protein, resulting in the exchange of GDP for GTP in the α subunit and subsequent dissociation of the α subunit from the βγ subunit. This α subunit then activates phospholipase C-β (PLCβ) to cleave membrane bound phosphatidylinositol(4,5)P₂ (PtdIns(4,5)P₂) 4 generating water soluble Ins(1,4,5)P₃ 1 and lipid soluble diacylglycerol (DAG) 5 (Scheme 1).

Scheme 1: Hydrolysis of PtdIns(4,5)P₂ 4 to Ins(1,4,5)P₃ 1 and DAG 5.

Tyrosine kinase linked receptors dimerize in response to stimuli, to allow ATP mediated autophosphorylation of intracellular tyrosine residues. In turn the Src homology region 2 domain of phospholipase C-γ (PLCγ) binds to the receptor, initiating activation of PLCγ via phosphorylation. Once activated, cleavage of PtdIns(4,5)P₂ 4 to Ins(1,4,5)P₃ 1 and DAG 5 occurs in the same way as shown in Scheme 1. The pathways then split into two branches, both playing crucial roles. Ins(1,4,5)P₃ 1 rapidly migrates across the cytoplasm of the cell where it binds to
InsP₃R’s on the surface of the ER/SR to initiate calcium release. Ins(1,4,5)P₃ 1 does however have a short lifespan, in the order of seconds before being either phosphorylated or de-phosphorylated by enzymes. Addition of a phosphate group is mediated by the calcium dependant kinase, phosphatidyl-3-OH kinase (PI(3)K), to generate inositol-1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄), which although its exact mechanism is unclear, does bind a specific GTPase-activating protein of the Ras family modulating further calcium release.² Some cellular responses initiated as a result of Ins(1,4,5)P₃ 1 calcium release are collated in Table 1.

<table>
<thead>
<tr>
<th>Target Tissue</th>
<th>Signalling Molecule</th>
<th>Major Response</th>
</tr>
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<tbody>
<tr>
<td>Smooth muscle</td>
<td>Acetylcholine</td>
<td>Contraction</td>
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<tr>
<td>Blood platelets</td>
<td>Thrombin</td>
<td>Aggregation</td>
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<tr>
<td>Hippocampus</td>
<td>Acetylcholine</td>
<td>Memory</td>
</tr>
<tr>
<td>Endocrine glands</td>
<td>Acetylcholine</td>
<td>Secretion</td>
</tr>
<tr>
<td>Mast cells</td>
<td>Antigen</td>
<td>Histamine secretion</td>
</tr>
</tbody>
</table>

Table 1: Overview of some cellular responses to Ins(1,4,5)P₃ 1.

The second product of the hydrolysis of PtdIns(4,5)P₂ 4, DAG 5 exerts different effects. First, the fatty acid chain can be cleaved, releasing arachidonic acid, which can undergo further modifications to achieve the eicosanoids. Second, and more importantly, a small rise in intracellular calcium associated with Ins(1,4,5)P₃ causes C-kinases (serine/threonine protein kinases) to translocate from the cytosol to the cytoplasmic face of the PM. Once achieved, stimulation with DAG 5 and calcium enables these kinases to phosphorylate selected serine or threonine residues on target proteins within the cell. Due to the rapid metabolism of DAG 5 it cannot sustain the required C-kinase activation for long term responses such as cell proliferation, therefore a secondary wave of DAG 5 is generated from the hydrolysis of PM bound phosphatidylcholine.

1.3 Intracellular calcium signalling – cADPR

Although Ins(1,4,5)P₃ 1 appears to operate as a universal calcium mobilising agent,³ it was considered that there may well be other messengers that operate within cells. Experiments using a robust sea urchin egg model showed the presence of other
calcium release mechanisms, which enhance and differentiate the signal, regulated by a family of pyridinium based nucleotide metabolites. By injection of NAD 6 and NADP 7, calcium release could be observed in microsomal egg fractions that were devoid of Ins(1,4,5)P3. This calcium efflux was observed from vesicles previously loaded by calcium ATPase’s, with the effect of NAD 6 seen as a cellular calcium release after a substantial delay, but NADP 7 appeared to initiate an immediate and potent calcium emission. Interestingly, calcium release initiated by addition of NAD 6 was only observed in a batch of Percoll purified microsomal fractions, incubated with supernatant or cytosol fractions from the same Percoll gradients, suggesting the requirement for a second soluble factor to enable the calcium release to occur. Two co-factors were in fact isolated to enable NAD 6 to produce calcium release. First was an enzyme responsible for the conversion of NAD 6 into the active metabolite, which was later identified as cADPR, the second, was calmodulin, that confers cADPR sensitivity. The instantaneous effect due to NADP 7 will be discussed in greater detail later in the chapter. These initial experiments lead a challenge to rationalise the exact structure and mechanism of action of this metabolite of NAD 6. Isolation and mass spectrometry of the product revealed that the structure was smaller than ADP-ribose by one water molecule, and in the same way that cAMP is 18 units lighter than AMP, it was recognised that this structure had in fact cyclised (Scheme 2).

**Scheme 2:** Cyclisation of NAD 6 into cADPR with NADase.

Clarification of the enzyme responsible for this conversion was then required. An endogenous NADase [E.C. 3.2.2.5] was known to hydrolyse NAD 6 into ADP-ribose, but generating an inactive metabolite from a crucial redox co-enzyme was considered
wasteful and thus not the only role for the enzyme. NADase was shown to catalyse
the formation of cADPR 2 from NAD 6 with such efficiency to cause complete
exhaustion of the endogenous cADPR calcium stores.\textsuperscript{12} As it was clear that this
enzyme, purified from Aplysia ovotestis, produced cADPR 2 and not ADP-ribose, it
was named ADP-ribosyl cyclase. In an effort to identify if there was a homologous
enzyme in the mammalian system, searching of the GenBank database revealed two
possibilities, CD38 and CD157, both of which are mammalian antigens.\textsuperscript{13} They were
shown to possess 30\% sequence homology with the Aplysia ADP-ribosyl cyclase and
are both capable of producing cADPR 2 from NAD 6 and interestingly, CD38 can
also hydrolyse cADPR to ADP-ribose. This suggests an inhibitory role of ADP-
ribose in channel modulation in eggs and smooth muscle cells,\textsuperscript{2,11} which is similar to
Ins(1,4,5)P\textsubscript{3} 1 in that the metabolite, Ins(1,3,4,5)P\textsubscript{4} has a cellular function. CD38 was
first isolated as a surface antigen on lymphocytes, but has since been shown to be
present in many other tissues including the eye and brain. Not only is it found on the
cell surface but also within selected organelles. There is however speculation that
cADPR 2 is formed in the extracellular space by PM CD38. Transportation of NAD 6
to the extracellular space may be via a conexin 43 hemichannel where it can then
undergo CD38 (ADP-ribosyl cyclase) catalysed conversion and translocation to the
intracellular space.\textsuperscript{2,14} Regardless of where cADPR 2 is synthesised, comparison of
CD38 knockout mice and wild-type mice has proven that in-vitro cADPR 2 synthesis
is massively impaired without the enzyme.\textsuperscript{15}

Experiments have shown that cADPR 2, when injected into sea urchin eggs, does
release calcium by activation of ryanodine receptors (RYR) located on the
endoplasmic reticulum (ER).\textsuperscript{2,13} Characterisation of this receptor has shown it to be a
large homotetrameric calcium release channel that is widely distributed among many
cell types.\textsuperscript{7} cADPR 2 induced calcium release is independent to that of Ins(1,4,5)P\textsubscript{3} 1,
because treatment with the Ins(1,4,5)P\textsubscript{3} 1 antagonist heparin does not desensitise the
response to cADPR 2. Antagonism is achieved by high concentrations of ryanodine
(~100 \textmu M) with millimolar concentrations of caffeine acting as an agonist.\textsuperscript{2} cADPR 2
plays an important role in mediating calcium induced calcium release (CICR) by
sensitising the RYR to sub-micromolar levels of calcium, thus increasing the
frequency of the ‘sparks’ leading to formation and propagation of the wave. Many
agonists of the cADPR 2 pathway are found to be cell permeant, such as nitric oxide, glucose and vitamin B12; however, cell impermeant agonists can act through interaction with the surface receptor. This is observed during activation of the T-cell/CD3 complex by the OKT3 antibody and also in longitudinal intestinal muscles by cholecystokinin, both causing calcium influx, stimulating ADP-ribosyl cyclase activity to elevate cADPR 2 levels. Alternative evidence however, suggests that activation of the cyclase may be a result of coupling to a G-protein as stimulation with a cell surface muscarinic agonist induces a 2-to 3-fold increase in cyclase activity that is inhibited with cholera toxin. This is in marked contrast to Ins(1,4,5)P3 whereby all activation appears to be via cell surface stimulation. This would suggest that the cell has employed two separate calcium messengers to distinguish between permeant and impermeant signals. There is however a conflicting report that cADPR 2 may not actually be an intracellular messenger as appreciable levels of cADPR exist in cells that do not express RYR's. This evidence may suggest that cADPR is a general regulator of calcium signalling, or possesses secondary roles discrete from calcium release.

A brief overview of the main functions of cADPR 2 is shown in Table 1.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Receptor</th>
<th>Cell</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>Muscarinic</td>
<td>Tracheal smooth muscle</td>
<td>Contraction</td>
</tr>
<tr>
<td>Anti-CD3 mAb</td>
<td>TCR/CD3</td>
<td>Jurkat</td>
<td>T cell activation</td>
</tr>
<tr>
<td>Cholecystokinin</td>
<td>CCKA</td>
<td>Pancreas: acinar cell</td>
<td>Secretion</td>
</tr>
<tr>
<td>(CCK)</td>
<td>Glucose kinase</td>
<td>Pancreas: β cell</td>
<td>Insulin secretion</td>
</tr>
</tbody>
</table>

Table 2: Overview of cADPR 2 activation pathways.

1.4 Intracellular calcium signalling – NAADP

Experiments injecting NADP 7 into sea urchin egg homogenate interestingly caused significant and rapid calcium release. This suggested that unlike NAD 6, enzymatic modification was not required and once analysed and separated by HPLC it became apparent that the release was in fact caused by a minor contaminant and not by NADP 7. Difficulties came when trying to decipher the structure, as 1H NMR spectroscopy generated a spectrum for NAADP 3 that was hardly distinguishable from that of NADP 7. Mass spectrometry did show that the structure was one mass unit heavier,
but it was not until eight years later that the structure of NAADP 3 was published (Scheme 3).\textsuperscript{16,17} Amazingly, this single functional group inter-conversion from amide to acid confers total specificity for the system as NADP 7 is completely inactive. Until that time, pyridinium based nucleotides were only thought to be involved in redox biochemistry, whereby the pyridinium ring is reduced to afford NADPH, whose primary role is to reduce fatty acids during their biosynthesis. Exploration was then performed to further understand its biological synthesis and significance.

![Scheme 3: Published structure and proposed mechanism of synthesis for NAADP 3.](image)

ADP-ribosyl cyclase [E.C. 3.2.2.5] from \textit{Aplysia californica}, homologous with CD38 had already been shown to catalyse the cyclisation of NAD 6, but it was also able to cyclise NADP 7 to produce cADPR-phosphate 10.\textsuperscript{1} Interestingly, this enzyme is also able to catalyse the replacement of nicotinamide for nicotinic acid at acidic pH. This base-exchange can be used to convert NAD 6 into NAAD 8 and NADP 7 into NAADP 3 (Scheme 3). This enzymatic synthesis of NAADP 3 is the only proposed mechanism for \textit{in-vivo} formation, however \textit{in-vitro} studies require high concentrations (mM) of nicotinic acid to be present with a pH of 4-5, which in cells is normally only observed in the lysosomes. It is also possible to drive the reverse reaction by exposing the cell to large quantities of cell permeable nicotinamide (IC\textsubscript{50} \sim 1.5 mM), thus achieving product inhibition by re-generation of β-NADP 7. Some feel that these reaction conditions are unlikely to exist within the cell and have speculated on the involvement of an NAAD kinase,\textsuperscript{14,16,17} or NADP 6 deaminase,\textsuperscript{17} although such enzymes are yet to be reported. A recent report using CD38 knockout mice supports the theory that CD38 is responsible for NAADP 3 synthesis. Analysis of brain tissue
homogenate from these animals showed that CD38 -/- tissue was unable to support NAADP 3 synthesis from NADP 7, although it is recognised that this does not categorically prove that the base-exchange reaction occurs in-vivo, it merely proves that CD38 is required for in-vitro NAADP 3 synthesis from NADP 7 using brain homogenate. Indeed, in this study, little attention is paid to non-base-exchange reactions.

NAADP 3 appears to interact with receptors that are discrete from those activated by Ins(1,4,5)P3 1 and cADPR 2 in sea urchin egg homogenates. It has also been found to be the most potent agonist of calcium channels with ranges of 10-100 nM stimulating the largest calcium signal. Interestingly, high concentrations (> 1-100 μM) fail to cause any detectable calcium release and sub-threshold concentrations totally desensitise sea urchin egg homogenates, such that subsequent challenge with maximal concentrations (10-100 nM) evoke no response. In other cell types such as rat brain, rat pancreatic acinar cells and T-cells activation occurs at approximately 1 μM, 50 nM and 100 nM (maximum) respectively. Inactivation also occurs with pancreatic cells and T-cells at 1-100 μM and 10 μM respectively, yet no inactivation at sub-threshold concentrations is observed.

Experiments using radiolabelled [32P] NAADP showed that binding, and hence retention of radioactivity was only observed in preparations where calcium release occurred. Once bound it was unaffected by either subsequent exposure to non-labelled NAADP, change in pH, or the presence of high calcium concentrations (10 mM). This corroborates the theory of irreversible binding, which may be linked to the self inactivation allowing speculation that, at least in sea urchin eggs, there is a possible 'one shot' calcium release mechanism, employed during fertilisation. [32P] NAADP binding was inhibited by NaCl in a completely reversible, and concentration dependant manner (0.5-1 M NaCl for a 25-75% reduction), and NaCl was able to reverse the inhibitory effect caused by sub-threshold NAADP 3 concentrations in sea urchin egg homogenates allowing maximal binding when re-exposed to [32P] NAADP. How this novel pathway interacts in the overall signalling cascade is still under intense scrutiny.
1.5 The synergy of calcium release

It is speculated that there is a close relationship between all three calcium release mechanisms and the term ‘channel chatter’ has been used to describe this. In pancreatic acinar cells and sea urchin eggs NAADP 3 acts to trigger the calcium response, which is subsequently propagated by Ins(1,4,5)P3 1, and cADPR 2, by CICR. However inhibition of either Ins(1,4,5)P3 1 or cADPR 2 blocks the NAADP 3 response in acinar cells (Figure 4(a)), yet in urchin eggs both receptors require inhibition suggesting a redundant pathway in that cell line (Figure 4(b)). Interestingly, in sperm cells and T-cell/CD3 complexes it seems to be more complicated whereby the NAADP receptors interlink with Ins(1,4,5)P3R in sperm and both Ins(1,4,5)P3R and RYR in T-cells.

Clearly the precise mechanism of coupling is still very poorly understood,1 but whether NAADP 3 does actually address a discrete calcium pool has still not categorically been proven. Experiments revealed that NAADP 3 can still cause calcium mobilisation after inhibition of calcium ATPase pumps by thapsigargin, which drains the ER calcium stores sensitive to Ins(1,4,5)P3 1 and cADPR 2. This shows that NAADP 3 does not mobilise calcium from the ER21,23 and later studies suggest that calcium is released from lysosome related organelles in sea urchin eggs, which will be discussed later.1,24 However a type 2 RYR from dog heart incorporated into a lipid bilayer showed a response to NAADP 3, although it should be acknowledged that the concentration was 100 times higher than previously reported for other systems. These findings may indicate that the NAADP receptor is an accessory protein, which binds to a known channel on the ER rather than an actual novel intracellular channel.14
There is evidence of spatial separation between stores activated by NAADP 3 and those by Ins(1,4,5)P3 1 and cADPR 2 found in starfish oocytes. Uniform photolysis of oocytes and pancreatic acinar cells preloaded with caged NAADP 3 showed a highly localised release of calcium in the region of the cortex and apical pole respectively, which would suggest a fine-tuned signal for a specific function. However, localised photolysis of caged NAADP 3 in sea urchin eggs produced a wave that propagated across the entire cell. There are however differences noted in starfish oocytes with a fluorescent halo, restricted to the cytosol, which rapidly decays when the NAADP is locally uncaged in Astropecten auranciacus cells. However, the wave initiated by activation of Ins(1,4,5)P3R, does eventually spread across the cell it just requires a longer time.

**1.6 Tissues responsive to NAADP**

Not as many cell systems have been identified as being responsive to NAADP 3 compared to those of cADPR 2. Table 3 lists a large selection of the systems where NAADP 3 has been shown to be required.
<table>
<thead>
<tr>
<th>Cell or tissue</th>
<th>NAADP effect</th>
<th>Effective concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cauliflower</td>
<td>Inactivation</td>
<td>3 nM</td>
</tr>
<tr>
<td></td>
<td>Activation</td>
<td>1 μM</td>
</tr>
<tr>
<td>Sea urchin egg</td>
<td>Inactivation</td>
<td>&lt;1 nM (IC(_{50}) = 200 pM)</td>
</tr>
<tr>
<td></td>
<td>Activation</td>
<td>&gt;3 nM (EC(_{50}) = 25 nM)</td>
</tr>
<tr>
<td>Starfish oocytes</td>
<td>Activation</td>
<td>&lt; ~1 μM</td>
</tr>
<tr>
<td>Frog neuromuscular junction</td>
<td>Activation</td>
<td>10 μM – 1 mM; liposomal delivery</td>
</tr>
<tr>
<td>Rat brain</td>
<td>Activation</td>
<td>EC(_{50}) = 1 μM</td>
</tr>
<tr>
<td>Rat pancreatic acinar cells</td>
<td>Inactivation</td>
<td>1-100 μM</td>
</tr>
<tr>
<td></td>
<td>Activation</td>
<td>50 nM</td>
</tr>
<tr>
<td>Rabbit heart</td>
<td>Activation</td>
<td>320 nM</td>
</tr>
<tr>
<td>Human T-lymphocytes (Jurkat)</td>
<td>Inactivation</td>
<td>10 μM</td>
</tr>
<tr>
<td></td>
<td>Activation</td>
<td>EC(_{50}) = 50 nM</td>
</tr>
<tr>
<td>Pancreatic β cells</td>
<td>Activation</td>
<td>~ 100 nM</td>
</tr>
</tbody>
</table>

Table 3: Overview of cells responsive to NAADP 3.\(^{20}\)

The mechanism of calcium release mediated by NAADP 3 has been extensively studied in a variety of cell types. NAADP 3 was first shown to mobilise calcium from sea urchin eggs\(^{16,17}\) and has since been shown to initiate and co-ordinate calcium signalling pathways.\(^1\)

**Sea urchin eggs**

Sea urchin eggs have become widely used as a tool for the investigation of calcium release as they can be produced in relatively large batches and possess multiple signalling mechanisms for calcium release, that are easily manipulated.\(^{26}\) In this way using caged compounds in combination with confocal microscopy, identification of the organelles targeted by NAADP 3 was obtained.\(^{24}\) It was shown that organelles disrupted via osmotic lysis using glycyglycyl-phenylalanine 2-naphthylamide (GPN, 100 μM), a substrate of the lysosomal exopeptidase cathepsin C, produced a significantly smaller rise in calcium when subjected to photo-activated NAADP 3.\(^{24}\) Lysosomes were fluorescence labelled with the targeted dye LysoTracker Red, whereupon introduction of GPN caused dissipation of the fluorescence, confirming their lysis. In contrast to this, GPN did not reduce the amount of calcium released by either
Ins(1,4,5)P₃ 1 or cADPR 2, suggesting that GPN-mediated disruption of lysosomes selectively reduces the response to NAADP 3.²⁴ It is known that protons are actively pumped into lysosomes through ATPase pumps and it is this gradient which drives the influx of calcium via a proton/calcium exchange pumps.²⁴ Bafilomycin A1 is a useful tool as it disrupts calcium storage through inhibition of the proton ATPase pump, which manifests itself as a reduced calcium release in response to NAADP 3 activation, for example, during fertilisation.²⁵

Fertilisation of the egg elicits a cell wide calcium wave, stimulating the initiation of cell division and eventual maturation.¹³ This can be visualised in Figure 5 by the localised photorelease of caged NAADP 3.

![Figure 5: Propagation of the calcium wave in a sea urchin egg upon localised photorelease of NAADP 3. The calcium released is visualised by its chelation to the fluorescent dye, Fluo-3.](image)

Until recently it was believed that immediately after fertilisation, the egg synthesised NAADP 3 or released it from a store to initiate the initial calcium release that eventually propagated across the entire cell as a wave by CICR.¹²¹ A recent study using the eggs of *Lytechinus pictus* (a sea urchin) showed binding of the spermatozoa to the egg plasma membrane (PM), may present a separate compartment containing NAADP 3. As a result, it was postulated that release of the sperm cytosol into the egg donated not only nuclear material, but also a locally high concentration of NAADP 3, which initiated the wave.²² There is however some contradictory evidence to suggest that the spermatozoa interacts with a NAADP receptor at the PM to initiate the synthesis of NAADP 3, however no such receptor has been isolated, yet NAADP 3 has been characterised in spermatozoa.²² Experiments revealed that egg homogenates pre-treated with sperm extract, or with an inhibitory concentration of NAADP 3 did not bind radiolabelled [³²P] NAADP. Furthermore, NAADP 3 and sperm extract were unable to displace radiolabelled [³²P] NAADP, but high salt concentration could displace both.²² These findings are consistent with the theory that spermatozoa
contain NAADP 3. It was also speculated that the base-exchange reaction was responsible in the spermatozoa for the conversion of NADP 7 to NAADP 3 (Scheme 3) as the absence of nicotinic acid inhibited its formation. The base-exchange reaction was time and pH dependent with maximal production after one hour and at pH 5, consistent with enzymatic formation. This evidence strongly suggests a role for NAADP 3 as an initiator of a cell wide calcium wave, once it is released into the cytosol of the egg during fertilisation. This in turn initiates downstream events leading to maturation of the cell.

**T-cells**

T-cell proliferation in response to an antigen has been attributed to activation of the T-cell/CD3 complex. This complex has been shown to elicit a fast increase in calcium caused by Ins(1,4,5)P$_3$ 1, followed by a sustained response mediated by cADPR 2.\textsuperscript{13,27} It is now emerging that NAADP 3 is closely linked to this activation pathway, but unlike sea urchin eggs, NAADP 3 at sub-threshold concentrations (10 nM) does not cause inactivation, but instead initiates low-amplitude calcium spikes.\textsuperscript{27} Stimulation with optimal concentrations (50 – 100 nM) triggered CICR from Ins(1,4,5)P$_3$ 1 and cADPR 2 stores. Interestingly, high concentrations (>10 μM) were self-inactivating, abolishing the calcium release arising from subsequent stimulation via the activated T-cell/CD3 complex, or Ins(1,4,5)P$_3$ 1 and cADPR 2 pathways.\textsuperscript{27}

**Pancreatic β-cells**

Calcium release and subsequent downstream insulin secretion has been studied in pancreatic β-cells. It has been shown that acetylcholine acts to stimulate Ins(1,4,5)P$_3$ 1 and cADPR 2 pathways through interaction with the ER and that glucose stimulates the NAADP 3 pathway mediated by lysosomal related organelles (Figure 6).\textsuperscript{25} This work showed that NAADP 3 mobilised calcium from a store independent from those stimulated by Ins(1,4,5)P$_3$ 1 and cADPR 2 on the ER. Using whole cells it was possible to demonstrate that glucose initiated calcium release lead to subsequent insulin secretion via an acidic lysosomal NAADP 3 store that was sensitive to bafilomycin A1.\textsuperscript{25} It is less clear how NAADP 3 elicits the influx of extracellular calcium into β-cells, however a possible mechanism may be linked to voltage operated channels similar to those observed in sea urchins. In response to external
stimuli, this direct release of calcium from internal stores, independent from those of other specific second messengers, confirms NAADP 3 as a second messenger for the first time. This rise in calcium has thus been shown to cause a downstream event and in this example causes the release of insulin to directly reduce the blood glucose concentration.

**Figure 6:** Effect of glucose causing NAADP 3 mediated calcium release from an organelle discrete from that acted on by cADPR 2 and Ins(1,4,5)P₃ 1.
1.7 Inactivation of NAADP

NAADP 3 has been shown to be highly active in a range of cell types, so there must be a highly selective mechanism of inactivation in order to switch off the signal (Scheme 4).

It has been shown in brain tissue that NAADP 3 is metabolised predominantly to NAAD 8 by dephosphorylation.\textsuperscript{28} This is in contrast to NADP 7 which mainly undergoes metabolism to ADP-ribose.\textsuperscript{28} Addition of the calcium chelator, EDTA showed attenuation of this metabolic pathway to form NAAD 8, revealing the intimate relationship between this feedback mechanism and cytosolic calcium concentration. These results also suggest that metabolism does not proceed via base-exchange as NAADP 3 is not converted to the NADP 7 metabolite ADP-ribose phosphate 10.\textsuperscript{28} Recently emerging data suggest that NAADP 3 might also be inactivated by reduction to NAADPH 9, catalysed by glucose-6-phosphate dehydrogenase [E.C. 1.1.1.49] with a small amount of ADP-ribose phosphate 10 also produced.\textsuperscript{29} NAADPH 9 had virtually no calcium release properties with
concentrations above 400 nM required to observe release. This result seems remarkable because one might expect some contamination or degradation of NAADPH to NAADP and concentrations as low as 3 nM (of 3) will cause calcium release. It was also shown that sub-threshold NAADPH concentrations, incubated for 5 minutes, serve to inhibit subsequent NAADP stimulation. However, in 5 minutes it would be reasonable to expect that the unstable NAADPH could undergo oxidation to NAADP, in sufficient concentration to cause the receptor inactivation, which would invalidate this result. It was speculated that NAADPH could either then be used as a precursor molecule to NAADP by undergoing oxidation before instigating calcium release, or as a metabolite that can efficiently inactivate the calcium signal allowing storage of this molecule in an inert state. Work undertaken within our group found that unlike NADPH, it was very difficult to produce NAADPH by either chemical or enzymatic methods, and even harder to purify due to decomposition to ADP-ribose phosphate, or re-oxidation to NAADP.

1.8 Summary of NAADP

NAADP has been shown to be active in a number of cell types producing an array of responses that have been characterised to varying degrees. It has now been firmly established as an endogenous molecule responsible primarily for the initiation and coordination of calcium release. Its release from stores independent of Ins(1,4,5)P and cADPR suggest that NAADP is a second messenger of immense interest. However, the exact mechanism for in-vivo synthesis and metabolism still remain a topic of huge debate. Unfortunately, studies are currently restricted due to the limited array of chemical probes available to interrogate this system. As the total synthesis of NAADP has not been previously published, analogues have been limited to direct alteration of NAADP or NADP modifications that still remain substrates for ADP-ribosyl cyclase. A disadvantage of this approach is potential contamination with very potent NAADP in the product. Total chemical synthesis of NAADP would provide a much more feasible route toward structurally relevant analogues. It is hoped that these analogues will help isolate the NAADP receptor protein and allow the in-vivo chemical pathway to be elucidated.
Chapter Two

Aims and objectives
2.1 Synthesis of chemical analogues

A small structure-activity relationship (SAR) of NAADP 3 (Figure 7) involving readily accessible modifications of NADP 7 such as substitution of nicotinic acid for analogues using Aplysia ADP-ribosyl cyclase had previously been investigated.\(^1\)

![Chemical structures and activity levels]

**Figure 7:** Activity of NAADP 3 and closely related analogues.

Installation of the alcohol equivalent 11 generated an inactive compound, which provided evidence that the carboxylate negative charge was essential for activity. This is confirmed by the fact that NADP 7 is totally inactive in the mobilisation of calcium. Altering the position of the carboxylic acid from the *meta* to the *para* position 12 also resulted in total loss of activity, suggesting that the active site of the receptor is very tightly controlled. There is however a small degree of tolerance of the negative charge or aromatic group as both the sulphonic acid 13 and quinoline 3-carboxylate 14 remain partially active. Pyridines substituted at the 2-position were not substrates for the cyclase enzyme inhibiting their inclusion into the SAR scheme (Figure 7).\(^1\)

Other analogues were obtained by conversion of the adenine base. Replacement of the adenine ring for a hypoxanthine (purinyl amine replaced by a hydroxyl) resulted in desamino NAADP 15 after base-exchange.\(^1\) Two fluorescent analogues were
synthesised by manipulation of commercially available etheno NADP. Replacement of the nicotinamide for nicotinic acid resulted in the formation of etheno NAADP 16 that with further modification led to etheno-aza NAADP 17 (Figure 7). Both were partially active but highly fluorescent, potentially enabling \textit{in-vivo} detection. Both of these analogues retained the nitrogen at the 6-position, suggesting that it may contribute to activity.30

NAAD 8 was completely inactive in mobilising calcium, while the 2',3'-cyclic 18 and the 3'-regio-isomers 19 were found to be less active, proving that the 2'-phosphate 3 is essential for activity.18 Formation of a phosphate ester at the 2'-position renders the molecule inactive, however the nitrophenyl ester 20 was synthesised to provide caged NAADP which could be activated \textit{in-vivo} upon flash photolysis (Figure 7).31

All of the analogues that confer activity are agonists and also retain the self-inactivation observed for NAADP 3. Despite this intriguing effect there has been no reported antagonists, even though agonists may be used as pseudo-antagonists \textit{via} the self-inactivation mechanism.17,32

This small study provided an invaluable insight into the chemical biology of NAADP 3; however the range is seriously limited by requiring substrates that can still be turned over by enzymes. This is emphasised by the lack of knowledge regarding other positions such as the positive charge and substitution at the 2-position of the pyridinium. Total chemical synthesis therefore offers the most practical approach to a wider range of analogues without the potential complication of trace NAADP contamination skewing observed result. Therefore formation of analogues, devoid of any potential trace of NAADP 3 would be of great value.

\textbf{2.2 Project aims}

The aim for this project is the development of a synthetic route that would be sufficiently flexible to allow modifications at key positions. Initially, these could focus on the pyridinium ring, but later targets include the adenosine-2'-phosphate and the pyrophosphate (Figure 8).
2.3 Synthetic strategy

Late stage pyrophosphate bond formation would enable the two halves of the molecule to be linked through a convergent synthesis, which should enable modifications to be installed in the initial stages of the synthesis and reduce potential problems that may be associated with late stage alterations. Chemical synthesis of the 2'-phosphate moiety would be required as biological studies have shown that the 3'-phosphate 19 is distinctly less active.\textsuperscript{18} To achieve this, an efficient synthetic scheme, incorporating minimal protecting groups would ideally be employed, thus avoiding potentially problematic late stage cleavage (Scheme 5).

Scheme 5: Retrosynthetic approach to the synthesis of NAADP.

The formation of nicotinic acid ribonucleotide will provide a number of synthetic challenges. Initially, glycosidic bond formation will be required, with a suitably protected pyridinium ester moiety that can be cleaved to afford the carboxylate during
the later stages of the synthesis and secondly, selective phosphorylation at the 5'-ribose hydroxyl will be required. To facilitate efficient synthesis, work will be carried out to achieve this without the use of ribose protecting groups, however, it may become necessary to incorporate these to afford the desired ribonucleotide.

Synthesis of the adenosine moiety will require manipulation of protecting groups as the 2'-ribose hydroxyl will need to be either selectively unveiled or activated to allow regio-specific phosphorylation to ensure purity of the final compound. Protected phosphates will also be required to increase the lipophilicity of the compound to enable reactions and purification to be carried out with organic solvents for a large proportion of the synthesis.

Once the 5'-hydroxyl has been phosphorylated, pyrophosphate bond formation will be required to link the two halves together. This will entail challenging chemistry as there is a limited pool of reported methods for this reaction and the yields are often poor. A method will need to be developed that offers high yields, as this is the critical step in the synthesis.

Once a synthetic route has been developed, the emphasis can shift towards analogue formation. Initially, modification to the pyridinium ring will enable further understanding of the importance of the positively charged nitrogen along with the necessity for the base labile glycosidic linkage. Once formed this analogue will be tested for its ability to release calcium in sea urchin egg homogenates to further develop the SAR surrounding NAADP 3.
Chapter Three

Synthesis of β-Nicotinamide Mononucleotide
3.1 Introduction

Development of a flexible route towards enantiomerically pure pyridinium ribosides was sought to enable the total chemical synthesis of NADP 7, NAADP 3 and an array of analogues. Phosphorylation at the 5'-position would then provide access to linkage with the adenosine moiety via a pyrophosphate bond, providing a late stage, convergent synthetic route. A flexible synthesis is required to enable the generation of analogues to investigate the biochemistry of NAADP 3.

β-Nicotinamide mononucleotide (β-NMN) 21 is a key portion of co-enzymes NAD 6 and NADP 7, allowing the transfer of hydrogen during redox reactions. In-vivo synthesis of NAD 6 proceeds by reaction of the vitamin niacin (nicotinate) 22, which is added to phosphoribosylpyrophosphate (PRPP) 23 to form β-nicotinic acid mononucleotide (β-NaMN) 24. Linkage to adenosine monophosphate (AMP), from adenosine triphosphate (ATP) 25, catalysed by nicotinamide/nicotinate mononucleotide adenylyltransferase (NMNAT) [E.C. 2.7.7.1], gives NAAD+ 8, which is finally converted to NAD 6 upon transfer of the amide group by exchanging glutamine into glutamate (Scheme 6).

\[
\text{PRPP} \quad \text{ATP} \quad \text{NMNAT} \quad \text{NAD}^+ \quad \text{Glu}
\]

\[
\text{PRPP} \quad \text{ATP} \quad \text{NAD}^+ \quad \text{Glu}
\]

Scheme 6: Biosynthetic pathway for synthesis of NAD 6.

In-vitro synthesis of N-1-pyridinium ribose compounds requires β-stereoselective glycosidic bond formation. Use of protecting groups is limited by requirement for subsequent cleavage in the presence of the newly formed labile pyridinium bond. Consequently, there have only been four main approaches reported over the past forty years.
β-NMN 21 has been produced by the enzymatic degradation of NAD 6, using NAD-pyrophosphatase.\textsuperscript{33} Reaction in the presence of enzyme adsorbed on buffered P-cellulose at 37°C provided 2.9 g of β-NMN 21 in 82 % yield based on NAD 6. This presents an attractive route to large-scale β-NMN 21 production, however it is impractical as a flexible route for the production of structural analogues.

Previous work used adenosine monophosphate (AMP) 26 as the starting material in the chemical synthesis of enantiomerically pure β-NMN 21 (Scheme 7).\textsuperscript{35} AMP 26 was degraded to ribose 5-phosphate 27 by refluxing in an aqueous solution of acidic Dowex resin. Subsequent treatment with dry ammonia gas afforded the 1-amino ribose 5-phosphate 28, which was followed by the in situ Zinke reaction with 1-(2,4-dinitrophenyl)-3-carbamoyl-pyridinium chloride (NDC) 29. This provided diastereotopic NMN 21 in quantitative yield from ribose-5-phosphate 27 in an isomeric ratio of 4:6 (α:β). Even though this route provides an efficient synthesis of β-NMN 21, it is not generated as the single diastereoisomer. However, as the next step required enzymatic coupling with NAD pyrophosphorylase [NADPP, EC 2.7.5.1] to ATP 25, resolution was not required due to the specificity of the enzyme for only the β-isomer.\textsuperscript{35}

\begin{align*}
\text{AMP} & \xrightarrow{i)} \text{Dowex H}^+, \text{water, } 100 \, ^\circ\text{C, } 6 \, \text{mins} \quad \text{AMP} \rightarrow \text{Ribose-5-phosphate} \\
\text{Ribose-5-phosphate} & \xrightarrow{ii)} \text{NH}_3, \text{ethylene glycol, ammonia gas, } 0 \, ^\circ\text{C, } 30 \, \text{mins, then} \, 27, \, 5 \, ^\circ\text{C, } 15 \, \text{h} \quad \text{Ribose-5-phosphate} \rightarrow 1\text{-amino ribose-5-phosphate} \\
\text{1-Amino ribose-5-phosphate} & \xrightarrow{iii)} \text{NDC, } \text{CH}_3\text{OH, } 5 \, ^\circ\text{C, } 14 \, \text{h} \quad \text{1-Amino ribose-5-phosphate} \rightarrow \text{NMN 21}
\end{align*}

\textbf{Scheme 7: Reagents and conditions:} i) Dowex H\textsuperscript{+}, water, 100 \, ^\circ\text{C, } 6 \, \text{mins}; ii) ethylene glycol, ammonia gas, 0 \, ^\circ\text{C, } 30 \, \text{mins, then} \, 27, \, 5 \, ^\circ\text{C, } 15 \, \text{h}; iii) NDC, CH\textsubscript{3}OH, 5 \, ^\circ\text{C, } 14 \, \text{h}.
A recent synthesis of NAD involved bromination of commercially available tetra-O-acetyl-β-D-ribose 30 using HBr to afford 31 (Scheme 8), prior to glycosylation with nicotinamide 32 to afford 33 in a 1:25 α:β ratio after re-crystallization. The reported deacetylation using methanolic ammonia caused substantial nucleoside cleavage. Low temperature conditions (-5 °C) solved this problem leading to pure β-nicotinamide nucleoside 34. Phosphorylation using an excess of POCl₃ and trimethylphosphate led to the formation of β-NMN 21 after isolation on Dowex resin.

\[ \text{HO-} \rightarrow \text{HO} \]

Scheme 8: Reagents and conditions: i) HBr, CH₂Cl₂; ii) nicotinamide 32, SO₂, -10 °C; iii) 3 equiv. of NH₃, 1 M in CH₃OH, -3 to -5 °C, 20 h; iv) POCl₃, PO(OMe)₃, -5 to 0 °C, 7 h.

Commercially available tetra-acetyl ribose 30 has also been activated with the Lewis acid trimethylsilyl trifluorosulphonate (TMSOTf) and reacted with nicotinamide 32 to afford the protected nicotinamide ribose 38. This reaction proceeded via the following mechanism. The anomeric acetate was eliminated via the formation of the oxonium ion 35, which underwent intramolecular ring closure initiated by the neighbouring 2-acetyl 36 to afford the α-oxonium cation 37. Subsequent reaction with nicotinamide 32 therefore provided the β-isomer 38 (Scheme 9). In situ transesterification was achieved by the addition of CH₃OH, which afforded nicotinamide nucleoside 39 as the β isomer, in >95 % yield as characterised by \(^1\)H
NMR spectroscopy, whilst avoiding basic protecting group removal, thus making it attractive and flexible synthetic route for \( \beta \)-NMN 21 and analogues.

Scheme 9: Reagents and conditions: i) Nicotinamide 32, TMSOTf, CH\(_3\)CN, 1 h; ii) CH\(_3\)OH, 30 mins.

### 3.2 Synthesis of nicotinamide riboside and its analogues

This efficient production of \( \beta \)-nicotinamide nucleoside 39 with mild acetate cleavage encouraged us to explore the Lewis acid activated route further. The formation of the tri-acetyl intermediate 38 was observed by TLC, but never isolated; instead the mixture was treated with CH\(_3\)OH to allow the in-situ transesterification to afford 39. Purification of the product on an activated charcoal column, as described in the literature,\(^{36}\) was unsuccessful due to the loss of material on the column. An alternative method of purification was thus sought. Partitioning of the reaction material between water and \( n \)-butanol, followed by silica flash chromatography of the concentrated aqueous phase, afforded the nucleoside triflate salt 39 in 91 \% yield as an oil (Scheme 9). Formation of the glycosidic bond was confirmed by analysis of the \(^1\)H NMR spectrum, which showed a downfield shift for the pyridinium protons suggesting that they were in the electron deficient environment of a positively charged nitrogen. By structural examination of the dihedral angle between the anomeric proton and the 2' proton it was possible to assign the stereochemistry from the \(^1\)H NMR spectrum. In ribose, the angle exists at approximately 130 to 140° when the anomeric proton is \( \alpha \) (hence substitution is \( \beta \)) (Figure 9). By substituting this angle into the Karplus equation it equates to a vicinal coupling of 4 to 5 Hz. The
corresponding value for $\alpha$ substitution would be approximately 8 Hz. Analysis of the anomeric proton indicated that the product had formed the single $\beta$-isomer with a coupling constant to the 2'-proton of 4.2 Hz. This successful result led us to explore how other pyridinium bases react under these conditions.

**Figure 9:** The dihedral angle between the anomeric proton and the 2' proton of $\beta$-D-ribose. Black = carbon; red = oxygen; blue = hydrogen.

The glycosylation was carried out using different pyridinium bases, initially methyl nicotinate and nicotinic acid, to ascertain the flexibility of the route (Scheme 10). Under the same conditions the desired methyl nicotinate riboside 40 was synthesised in 81 % yield after partition between water and $n$-butanol followed by flash chromatography of the concentrated aqueous phase. Using nicotinic acid, however the reaction failed and the only material purified from the reaction was nicotinic acid and deacetylated ribose.

**Scheme 10:** *Reagents and conditions:* i) a) Methyl nicotinate, TMSOTf, CH$_3$CN, 1 h; b) CH$_3$OH, 30 mins; ii) nicotinic acid, TMSOTf, CH$_3$CN, 2 h; b) CH$_3$OH, 30 mins; iii) PSL, 60 °C, 24 h, or 1 M HCl, 18 h.

Analysis of the $^1$H NMR spectrum for the methyl ester 40 proved that glycosylation had occurred, as shown by a downfield shift of the pyridinium protons, while the $\beta$-stereochemistry was confirmed by examination of the anomeric coupling constant
Even though hydrolysis to the carboxylic acid 41 at this stage in the synthesis is not required, trial reactions would ascertain whether hydrolysis may be possible and if so, highlight any potential problems that could be encountered later in the synthesis (Scheme 10). Basic hydrolysis was avoided though, due to possible epimerisation to 42 or cleavage of the glycosidic link (43 and 22), as previously reported (Scheme 11).37

Scheme 11: Possible results of using aqueous base to perform the ester hydrolysis.

A method utilising an enzyme was investigated using Pseudomonas cepacia lipase [E.C. 3.1.1.3] at 60 °C over 24 hours,38 but unfortunately this proved to be unsuccessful. Analysis of the 1H NMR spectrum showed that both the anomeric and pyridinium protons had shifted upfield, presumably due to glycosidic bond cleavage, leaving an impure mixture of ribose 43 and nicotinate 22. Acid catalysed ester hydrolysis using 1 M HCl was also evaluated, but this failed to show that a reaction occurred in all but one instance, when the 1H NMR spectrum did show the presence of a second anomeric peak further upfield at 6.27 ppm. It was not possible to isolate this product to obtain any reliable data from this reaction and the focus was therefore shifted towards a protecting group, such as a benzyl ester that could undergo late stage neutral cleavage using H2, without causing glycosidic bond hydrolysis or other side reactions.

Initially nicotinic acid was protected using 0.9 equivalents of benzyl alcohol in the presence of DCC and DMAP to afford the ester 44 as a clear colourless oil in 67% yield. The ester 44 was then reacted with tetraacetyl-ribose 30 under the established protocol (Scheme 12). As the product was soluble in both water and n-butanol, separation was accomplished using H2O and EtOAc, and the concentrated aqueous phase later analysed by 1H NMR spectroscopy. An anomeric peak at 5.9 ppm (J=3.9 Hz, characteristic of the β-isomer), and the pyridinium protons confirmed that
glycosylation had occurred. However, no signals were observed for the benzylic ester protons. Mass spectrometry analysis showed a peak at 256 establishing that the nicotinic acid riboside 41 had indeed been formed (Scheme 12) presumably via in situ TMS catalysed debenzylation in 87 % yield. For the complete synthesis of NAADP the carboxylic acid is required, but it must remain protected until the final stages to prevent unwanted side reactions. Avoiding an aqueous workup and subjecting the crude material to silica flash chromatography, afforded the benzyl protected material 45 in 82 % yield, although, it still remained extremely water sensitive, and hence unusable in the synthetic scheme.

Scheme 12: Reagents and conditions: i) a) Benzyl nicotinate 44, TMSOTf, CH$_3$CN, 2 h; b) CH$_3$OH, 1 h; ii) aqueous work up.

3.3 Phosphorylation of nicotinamide riboside and its analogues

Conditions were sought to achieve phosphorylation of the nicotinamide ribose 5'-alcohol 39. Methods utilising either trimethyl or triethyl phosphate should allow the selective phosphorylation without the need for protecting groups based on precedence for phosphorylation of guanosine 46. For guanosine, the 5'-hydroxyl group is believed to form a hydrogen bond with the P=O group, which acts as a Lewis base, while the N$^9$ interacts with the phosphorus atom (Figure 10). It was speculated that nicotinamide riboside 39 would behave in a similar manner to enable the selective phosphorylation, but several attempts to treat nicotinamide riboside with stoichiometric quantities phosphorus oxychloride (POCl$_3$) in triethylphosphate produced no visible results.
Figure 10: The complex formed between guanosine and triethylphosphate causing the activation of the 5' hydroxyl.

The addition of 0.5 equivalents of H$_2$O to POCI$_3$ was explored as this produces a more reactive species that may provide better yields (Scheme 13). Monitoring of the reaction was difficult due to the high polarity of both the starting material and product. Conditions for reverse phase HPLC were found, enabling a change in retention time to be detected (Method B: R$_T$ 8 minutes (39) to 3 minutes (21)). Purification of the β-NMN 21 from this reaction mixture proved difficult due to the polarity of the products. Reverse phase chromatography failed to separate the compounds as they eluted on the solvent front when loaded in H$_2$O. Ion exchange chromatography was also difficult using positively charged resin, such as AG-MP1 as neither the neutral product 21, nor cationic starting material 39, was retained. Previous literature purifications of β-NMN 21 utilised two successive negatively charged resins, Dowex 1X2 formate and 50 WX8 H$^+$ respectively. Unfortunately, attempts at this method only yielded triethylphosphate.

Scheme 13: Reagents and conditions: i) Trimethylphosphate, POCI$_3$, H$_2$O, 5 h, 0 °C.

Ultimately, it was found that using trimethylphosphate provided better yields, perhaps enabling formation of the complex as shown in Figure 10. Precipitation of the product from CH$_3$CN:diethylether (1:3), followed by elution through the same negatively charged resins provided β-NMN 21 in 14 % yield. This yield does not correlate with the conversion observed by HPLC analysis, leading us to believe that
loss during purification had occurred. Even though this yield is low, it does enable the synthesis to continue to attempt the formation of protected acid ribonucleotides. Improvements to the yield could be achieved by the development of a negatively charged resin purification method that would retain the starting material 39 allowing separation from the neutral product 21.

Phosphorylation of the benzyl and methyl nicotinate (45, 40) riboside led to a complex mixture of products (as shown by both $^1$H and $^{13}$C NMR), which could not be purified. Attempts to precipitate the products via a variety of conditions only led to product degradation. Phosphorylation of the nicotinic acid riboside 41 gave a product that proved just as difficult to purify even though a precipitate was obtained (Scheme 14). This method was therefore abandoned as a route to install the 5’-phosphate on protected nicotinic acid ribosides.

Scheme 14: Reagents and conditions: i) Trimethylphosphate, POCl$_3$, H$_2$O, 5 h, 0 °C.

An alternative route could involve synthesis of ribose 5-phosphate, followed by formation of the glycosidic bond, to afford either $\beta$-NMN 21, or protected carboxylate analogues. POCl$_3$ could be utilised to install the 5’ phosphate, but phosphoramidite chemistry may provide a more efficient method of producing a protected phosphate that may be easier to purify using silica flash chromatography. Protecting groups on the three secondary alcohols would ensure regio-selectivity and may further increase lipophilicity, thus aiding organic solubility and purification.
3.4 Formation of β-D-ribose 5-phosphate

D-Ribose 43 was selectively protected using trityl chloride and pyridine exploiting the higher reactivity of the 5'-alcohol leading to 47 in 67 % yield, after purification (Scheme 15). Mass spectrometry analysis only showed a peak at 243 due to cleavage of the labile trityl group. Acetylation with acetic anhydride and Et₃N provided tri-acetyl ribose 48 in 69 % yield (Scheme 15). The coupling constant of the anomeric proton (1.0 Hz) in the ¹H NMR spectrum indicated the acetate protection had adopted the β-conformation, even though the dihedral angle must be strained to approximately 110°. This is further supported in the literature, which confirms that Et₃N does afford primarily the β-isomer at 6.22 ppm. When pyridine is used as a catalyst both isomers are produced in a 1:1 ratio and the α isomer is further downfield at 6.53 ppm. As no peak is observed in that region, it was concluded that the β-isomer had been selectively formed. Trityl deprotection using a 2 % solution of TFA in CH₂Cl₂ (Scheme 16) initially led to only partial cleavage of the trityl group due to the rapid back reaction with the reactive carbocation species. 2 % H₂O was added to scavenge this species leading to the improved yield of 76 % of triacetyl-ribose 49 after purification.

The tri-acetyl ribose 49 was then reacted with POCl₃ under the standard protocol to phosphorylate the primary hydroxyl (Scheme 17).
Scheme 17: **Reagents and conditions:** i) Trimethyl phosphate, POCl₃, H₂O, 0 °C, 5 h.

As the triacetyl-ribose 49 no longer possessed a chromophore, TLC visualisation by UV light was difficult, but staining with PMA or KMnO₄ revealed the disappearance of starting material 49 and the appearance of a new spot on the baseline. Extraction of the product 50 into water afforded a mixture that was difficult to resolve. Purification by AG-MP1 chromatography was thought possible as the product was negatively charged. However, the poor UV absorbance meant that each fraction required staining with PMA, but isolation only afforded trimethyl phosphate. As the remaining hydroxyls were protected, phosphoramidite chemistry was employed to provide the desired di-benzyl protected 5'-phosphate, which contained a UV active chromophore that also aided in detection and purification. Reaction of the primary alcohol 49 with the benzyl phosphitylating agent 52 and imidazolium triflate 51 catalyst afforded, after mCPBA oxidation, the desired protected phosphate 53 in 85 % yield following silica flash chromatography (Scheme 18).

![Diagram of chemical reactions](attachment:diagram.png)

**Scheme 18:** **Reagents and conditions:** i) a) Bis(benzyloxy)diisopropyl aminophosphine 52, imidazolium triflate 51, CH₂Cl₂, 3 h; b) mCPBA, -78 °C, 30 mins.

Attempts to remove both benzyl protecting groups under hydrogenation conditions in the presence of 20 % Pd(OH)₂/C and cyclohexene proved difficult. Despite both TLC analysis and ³¹P NMR spectroscopy (δ 0.69 ppm) showing complete reaction within three hours a complicated mixture of products was obtained after purification. A peak at 355 by mass spectrometry confirmed that the free phosphate 50 had indeed been formed (Scheme 19), so this material was therefore used crude in the following steps.
Both the benzyl protected 53 and crude free phosphate 50, were subjected to glycosylation with nicotinamide 32 using the established protocol. It was speculated that if CH₃OH, in the presence of TMSOTf cleaved the acetyl protection, it may replace the benzyl groups with methyl esters 54 (Scheme 20).

With this in mind both CH₃OH and water were used in two separate reactions to perform the in-situ acetyl cleavage for the benzyl protected material 55. As there was no risk of forming the methyl ester in the reaction with the free phosphate 50 only CH₃OH was used to perform the deacetylation.

In all three cases ¹H NMR spectroscopy showed the reaction had failed as no anomeric and downfield nicotinamide protons were observed. After work up, neither ribose moieties could be recovered or adequately characterised to establish the result of the reaction (Scheme 21).
**3.5 Summary**

Overall, the Lewis acid catalysed route enabled the synthesis of nicotinamide ribosides, but further experimentation is required to develop structural analogues and to explore the phosphorylation. An alternative strategy toward late stage installation of the nicotinamide led to a synthesis of pyridinium ribose 5'-phosphate, but further work will be required to progress the N-glycosylation. Glycosylation could be carried out on tetra-protected ribose 48 (Scheme 22), as the product formation could be easily monitored by TLC with purification on silica flash chromatography possible. The material could then be selectively deprotected to provide the 5'-alcohol 57, followed by phosphitylation and hydrogenation of the resulting dibenzyl phosphate to afford β-NMN 21. Purification of these types of compound is still a problem and to further this area of work development of an adequate purification protocol, such as using a negatively charged resin needs to be undertaken.

**Scheme 21:** *Reagents and conditions:* i) a) Nicotinamide, TMSOTf, CH$_3$CN, 2 h; b) CH$_3$OH, 1 h; ii) a) nicotinamide, TMSOTf, CH$_3$CN, 2 h; b) H$_2$O, 1 h; iii) a) nicotinamide, TMSOTf, CH$_3$CN, 2 h; b) CH$_3$OH, 1 h.

**Scheme 22:** Representation of the overall synthetic route to β-NMN 21.
Chapter Four

Selective adenosine phosphorylation
and pyrophosphate formation
4.1 Selective diphosphate adenosine formation

4.1.1 Introduction

The synthesis of NADP 7 and NAADP 3 could be achieved by pyrophosphate bond formation between a suitably protected 2',5'-adenosine bisphosphate and β-NMN 21 or β-NaMN 24. This approach required the development of a method to selectively install phosphates onto the 2'-and 5'-hydroxyls of adenosine without cyclisation, or subsequent migration to the 3'-hydroxyl.

Methods to selectively modify the 2'-position of adenosine 58 include routes to either selectively activate one hydroxyl (Scheme 23), or achieve protection of the remaining hydroxyls (Scheme 24 and Scheme 25).

![Diagrams showing the synthesis of NADP and NAADP](image)

**Scheme 23: Reagents and conditions:** i) Dibutyltin oxide, CH₃OH, reflux, 30 mins; ii) tosyl chloride, Et₃N, 5 mins; iii) benzoyl chloride, Et₃N, 15 mins; iv) hexabutyldistannoxane, POCl₃, 20 mins.

Formation of the stannylene acetal 59 has been investigated as a method to selectively functionalise the 2'-hydroxyl. Reaction with tosyl chloride led to the single regio-isomer 60. The observed regioselectivity was attributed to the formation of the dimeric structure 59, which forces the oxygen atoms to adopt either an apical or equatorial position. The apical oxygen is more reactive, since the equatorial position is deactivated by coordination to the tin atom. However, benzoylation afforded a mixture of regio-isomers, which when crystallised yielded primarily the 3'-product 61. Whether this was due to ring opening of the acetal at the 3' position or that the 3'
Regio-isomer was thermodynamically more stable was unclear. Subsequent attempts to selectively install a protected phosphate also produced a mixture of 2'-and 3'-products 62 (1:9) (Scheme 23).

This method of selective activation was attempted but as a result of failed tosylation later abandoned. As a result subsequent phosphorylation was never attempted. However, on reflection this approach could have been revisited had time permitted as it provides a fast and efficient synthesis of phosphorylated adenosine.

Regioselective deprotection of benzoyl protected adenosine 63 has been explored to selectively release the 2'-hydroxyl (Scheme 24). Previous work utilised either hydrazine hydrate to afford pure 2'-hydroxyl 64 in 64 % yield, or KO'Bu to achieve a mixture, whereby the prominent species was the exposed 2'-hydroxyl 64 (96 %, 5:1; 2':3'-hydroxyl). The regioselectivity was attributed to the greater acidity of the 2'-hydroxyl group, and X-ray crystallography of purine ribosides showed the C(2')–O(2') to be shorter than the other two hydroxyl C-O bonds, suggesting it may behave as the most active towards nucleophiles such as hydrazine hydrate and KO'Bu.

Tri-O-benzoyl adenosine 63 was synthesised to explore the selective cleavage of the 2'-benzoyl protection (64). Unfortunately, our attempts only led to a mixture of products that were inseparable by flash chromatography. This caused us to explore a further method that avoided a two-step protocol, and would allow unambiguous exposure of the 2'-hydroxyl.

A protecting group that simultaneously tethers the 3' and 5'-hydroxyls of adenosine 58 that leaves the 2' hydroxyl free for unambiguous modification has been established (65). Introduction of the 1,3-dichloro-1,1,3,3-tetraisopropyl disiloxane protecting group allowed selective formation of 2'-thioadenosine 66 and 2'-deoxynucleotides.
which has since become an established route for the synthesis of DNA (Scheme 25).

Scheme 25: Reagents and conditions: i) 1,3-Dichloro-1,1,3,3-tetraisopropyl disiloxane, pyridine, 3 h.

4.1.2 Synthesis of 2', 5' phosphorylated adenosine

Adenosine 58 was reacted with one equivalent of 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane in pyridine for three hours to afford the tetraisopropyldisiloxane (TIPDS) adenosine 65 in an 80 % yield (Scheme 25).45 Synthesis of a suitably protected phosphitylating reagent was required to install the protected phosphate at the 2'-hydroxyl. Diisopropylamine and phosphorus trichloride (PCl3) were reacted to afford the chlorophosphine 68 in 71 % yield.48 Substitution with two equivalents of hydroxypropionitrile afforded the phosphitylating reagent 69, in 74 % yield after flash chromatography on silica gel, pre-treated with Et3N (Scheme 26).49 It was important to ensure that the excess Et3N was completely eluted to prevent unwanted base-induced cyanoethyl elimination.

Activation of the phosphitylating reagent required displacement of the diisopropylamine with a suitable azole catalyst. If tetrazole is used (pKₐ ~ 5), phosphitylation would also occur on the purinyl amine.50,51 However, the analogous imidazolium phosphane 70 (imidazole pKₐ ~ 7) is less reactive enabling phosphitylation solely at the free hydroxyl.50 Therefore, the phosphoramidite 69 was treated with imidazolium triflate 51 to yield the imidazolium phosphine 70, which was reacted with protected adenosine 65, and subsequently oxidised with mCPBA to
afford the 2'-di(2-cyanoethyloxy) phosphate adenosine derivative 71 in 98% yield (Scheme 26).

Scheme 26: Reagents and conditions: i) Hydroxypropionitrile, Et$_3$N, CH$_2$Cl$_2$, -78 °C to RT, 2 h; ii) imidazolium triflate; iii) a) bis(2-cyanoethoxy) diisopropylamino phosphine, CH$_2$Cl$_2$, 3 h; b) mCPBA, -78 °C, 30 mins.

Selective protodesilylation of the TIPDS protecting group had previously been explored using 0.2 M HCl in dioxane–H$_2$O, 1 M HCl in dioxane or HF–pyridine. However, under these conditions, total deprotection also occurs and yields are moderate.$^52$ With dilute TFA (TFA–H$_2$O–THF (1:1:4; v/v/v)) however, ring opening occurs exclusively at the less hindered 5'-silyl-ether in high yield.$^52$ Reaction of 71 for three hours at 0 °C under these acidic conditions afforded the adenosine derivative with a free primary hydroxyl 72 in quantitative yield (Scheme 27).

Scheme 27: Reagents and conditions: i) TFA–H$_2$O–THF (1:1:4; v/v/v), 0 °C, 3 h; ii) a) bis(benzyl)diisopropylaminophosphine, imidazolium triflate, CH$_2$Cl$_2$, 18 h; b) mCPBA, -78 °C.
Phosphoramidite chemistry was again used to install the second phosphate. However, a second phosphitylating reagent possessing orthogonal protecting groups to the rest of the molecule was required to enable later selective cleavage. Bisbenzylphosphine 52 was synthesised in 79% yield following the standard protocol.53 Subsequent activation with imidazolium triflate 51 and reaction with the deprotected primary hydroxyl 72 led to the formation of the phosphite, but this required a greater reaction time than the 2'-phosphitylation, presumably due to increased steric hindrance. Overnight reaction followed by mCPBA oxidation, afforded the bis-phosphate 73 in a satisfactory 76% yield after flash chromatography (Scheme 27).

The final synthetic step, in the preparation of the pyrophosphate bond formation, was to selectively remove the 5'-phosphate benzyl groups to afford the free phosphate 74. Retention of 3'-and 2'-phosphate protecting groups was desirable in order to reduce the risk of phosphate migration, or spurious pyrophosphate formation at the 2'-phosphate.

Scheme 28: Reagents and conditions: i) 20% Pd(OH)$_2$/C, cyclohexene, CH$_3$OH, H$_2$O, 80 °C, 3 h.

Reductive deprotection using 10% Pd/C and hydrogen at 50 °C proved to be successful for the removal of the benzyl protection and was accompanied by a downfield shift in the $^{31}$P NMR spectrum (-0.42 ppm to 1.5 ppm). However, difficulties were encountered in the removal of the catalyst. Initially the compound adhered to celite, but this problem was overcome using a membrane filter. However, the resulting product was black, suggesting palladium contamination. Subsequent treatment with chellex resin sought to remove any remaining metal ions, but analysis by $^1$H NMR spectroscopy indicated a complex mixture, betrayed by a second anomeric peak δ 0.06 ppm upfield of the major peak. Exploration of the literature
revealed that adenosine compounds had been cleanly deprotected previously using 20% Pd(OH)$_2$/C and cyclohexene as a source of hydrogen.\textsuperscript{54} Reaction in CH$_3$OH and water at 80 °C for three hours, followed by membrane filtration, afforded clean, deprotected phosphate 74 in 82% yield without the need for flash chromatography (Scheme 28).

With this final step completed, a high yielding (49% yield, over five steps) and robust, synthetic route to a protected 2',5'-adenosine diphosphate 74 in had been developed. This could be carried forward to investigate the formation of the pyrophosphate bond and subsequent protecting group removal.

### 4.2 Pyrophosphate bond formation

#### 4.2.1 Introduction

Pyrophosphate bond formation is often difficult to achieve because of slow reaction times and the challenging purification of the water-soluble products leading to poor yields. Current literature procedures for pyrophosphate bond formation activate one of the phosphates to enhance its electrophilic properties, and the common methods are described below.

An early synthesis of NAD\textsubscript{6} utilised a vast excess of dicyclohexylcarbodi-imide to condense racemic NMN 21 and AMP 26. Isolation was achieved by selective enzyme reduction of the β-form by alcohol dehydrogenase, followed by alkaline destruction of the α-form and subsequent enzymatic oxidation to afford the correct β-isomer of NAD\textsubscript{6}.\textsuperscript{55,56} By using mixed 2',5'-and 3',5'-adenosine diphosphates, prepared from adenosine, they also tried to synthesise NADP. Unfortunately the material was contaminated with β-NMN 21 and adenosine diphosphate after purification. It was believed that this was due the apparent instability of NADP when the phosphate is carried at the 3'-position.\textsuperscript{55} This would however have provided a method for the synthesis of regio-selective NADP 7, if they could separated the two regio-ismoers.

Previous NAD\textsubscript{6} syntheses have activated adenosine monophosphate (AMP) 26 as an electrophile using the phospho-imidazolide, triazolide or tetrazolide.\textsuperscript{37} Activation of β-NMN 21 via the phospho-imidazolide was also explored, but all these routes
yielded NAD 6 in less than 5%. It was reasoned that the low reactivity of β-NMN 21 was a consequence of inner salt formation, making it less nucleophilic. Accordingly, activation of AMP-imidazolide and β-NMN 21 using the Lewis acid MnCl₂·4H₂O afforded NAD 6 in an improved, 25% yield. With further fine-tuning, using AMP-morpholidate 75, a respectable 58% yield was achieved. Interestingly, the authors claim that MnCl₂ was most active when stored in formamide, over 4Å molecular sieves for three days. Anhydrous MnCl₂ was found to be inferior and the identity of the active species remains unclear.

Alternative literature examples explore the production of various sugar-base pyrophosphate couplings using acid catalysts to accelerate the phosphomorpholidate chemistry. Kinetic investigations of various different acids such as 1,2,4-triazole, acetic acid, N-hydroxsuccinimide and 1H-tetrazole, showed that 1H-tetrazole was the most efficient catalyst providing pyrophosphate synthesis in 76-91% isolated yields, with reactions complete after two days. It was concluded that 1H-tetrazole not only acted as an acid, protonating the morpholine nitrogen 76, but also as a nucleophilic catalyst. This allowed nucleophilic substitution to the more reactive phosphotetrazolide 77, which underwent coupling to the second free phosphate 78, forming the pyrophosphate 79 in 76% yield (Scheme 29).

\[
\text{Scheme 29: Formation of the phosphotetrazolide. Reagents and conditions: i) 1H-tetrazole, pyridine, 78 2 days.}
\]
Phosphate activation via the formation of the imidazolide represents an alternative strategy that has been incorporated into the syntheses of a uridine dinucleotide and an oestrogen sulphotransferase inhibitor. Addition of carbonyl diimidazole (CDI) to uridine monophosphate (UMP) generated the activated imidazolium phosphite, which when treated with a second phosphate formed the pyrophosphate bond (Scheme 30) in acceptable yield. The oestrogen sulphotransferase inhibitor was synthesised in 43 % yield (not shown).

Further work examined the formation of a pyrophosphate bond between UMP morpholidate and imidazolide with galactofuranose to form the anti-microbial UDP-α-D-galactofuranose. Unfortunately, reaction times were modest (1-2 days) and the observed completion, poor (50 %) with corresponding low yields (19-23 %). UMP was activated using trifluoroacetic anhydride (TFAA) towards a more efficient route leading to the mixed anhydride and simultaneous esterification of the remaining hydroxyls, providing an intermediate that was soluble in DMF (Scheme 31). Subsequent addition of 1-methylimidazolide afforded the highly reactive methylimidazolide-phosphine, which could then be coupled to a second free phosphate to generate the pyrophosphate in an apparently quantitative conversion as observed by 31P NMR spectroscopy. Reaction times were very short (2h, 0 °C), but purification proved difficult and isolated yields were only 30-40 %.
Scheme 31: Formation of the methylimidazolide. Reagents and conditions: i) a) Dimethylaniline, Et$_3$N, CH$_3$CN, 0 °C; b) added dropwise, TFAA, CH$_3$CN, 0 °C, 5 mins; ii) 1-methylimidazole, Et$_3$N, CH$_3$CN, 0 °C, 5-10 mins

4.2.2 Pyrophosphate synthesis

We initially explored the well established morpholidate chemistry, which lead us to revisit, the reported synthesis of NAD 6.\textsuperscript{37} We chose to generate commercially available AMP-morpholidate 75 from the free acid of AMP 26.\textsuperscript{61} Once formed, ($\delta_p$ 8.77), the AMP-morpholidate 75 was precipitated by the addition 0.1 M NaI in acetone in quantitative yield. This was then reacted with $\beta$-NMN 21 using 0.2 M MnCl$_2$ in formamide to afford NAD 6 in 27 % yield after ion exchange chromatography (Scheme 32).

Scheme 32: Reagents and conditions: i) Powdered triphenylphosphine, 2,2'-dipyridyldisulphide, morpholine, DMSO, 4 h; ii) 0.2 M MnCl$_2$ in formamide (stored over 4 Å molecular sieves for three days), MgSO$_4\cdot7$H$_2$O, 18 h.
This methodology was transferred directly to the modified adenosine phosphate 74 and synthesis of the activated morpholidate was attempted. *In-situ* analysis by $^{31}$P NMR spectroscopy ($\delta$ 8.0 ppm) showed the reaction proceeding as expected, but purification proved difficult. Eventually, crude material was used to form the pyrophosphate bond, but analysis by HPLC and $^{31}$P NMR spectroscopy showed that no reaction had taken place. This was reasoned to be a result of the poor nucleophilicity of $\beta$-NMN 21. Previous work circumvented this poor reactivity by employing a Lewis acid, but this did not work for our target. We considered that activation of $\beta$-NMN 21 as the electrophile component would provide a more reactive, organic soluble starting material.

Using the method reported for the synthesis of UDP-$\alpha$-D-galactofuranose 86, generation the $\beta$-NMN methylimidazolide, via formation of the trifluoroacetyl mixed anhydride was explored. Initial phospho-methylimidazolide activation proceeded with the observation of a singlet at $\delta$ –10 ppm in $^{31}$P NMR spectrum. Disappointingly, addition of the adenosine analogue 74 did not result in the characteristic pyrophosphate signal in the $^{31}$P NMR spectrum and no product was obtained. This reaction was repeated using a variety of model reactants, including coupling between adenosine and cytosine monophosphate, but a pyrophosphate was never isolated from these mixtures. This method was therefore abandoned, although activation of $\beta$-NMN 21 was still regarded as essential.

![Diagram](image)

**Scheme 33:** Formation of the activated $\beta$-NMN via the mixed anhydride to the imidazolide with $^{31}$P NMR spectra values.

We turned our attention to potentially more simple phosphate activation using carbonyl diimidazole (CDI). Activation of $\beta$-NMN 21 occurs in two stages; first the
mixed anhydride is formed 83, followed by subsequent substitution to the imidazolide 84 (Scheme 33). Initial mixed anhydride 83 formation occurred within forty-five minutes and was accompanied by solubilization of β-NMN 21 in DMF and a shift in the $^{31}$P NMR spectrum from approximately 1 ppm to -9 ppm. The second stage was harder to detect by $^{31}$P NMR spectroscopy as the chemical shift is only about 0.8 ppm upfield, but after three hours phospho-imidazolide 84 formation was complete (Figure 11). CH$_3$OH was introduced to quench any remaining carbonyl diimidazole, before the addition of the protected adenosine 74 (Scheme 34).

**Chemical shift of β-NMN 21.**

After 45 mins the transition to the mixed anhydride 83 can be observed.

After two hours the reactive imidazolide 84 is partially formed.

After three hours the imidazolide 84 is formed and the second phosphate can be added.

The pyro-phosphate signal was observed after 18 h. (The second peak is the 2' adenosine phosphate at -2.7 ppm).

**Figure 11: NMR spectra to show the chemical shift during the pyrophosphate bond formation.** Shows reaction drawn in Scheme 34.
The $^{31}$P NMR spectrum showed consumption of both the 5'-adenosine phosphate 74 and the activated β-NMN 84 through the production of the characteristic pyrophosphate multiplet at -10 to -11 ppm (Figure 11). Analysis using ion exchange HPLC (AG-MP1) showed the appearance of a new peak at 11 minutes with good separation from any impurities. This retention time was longer than expected and analysis of the crude mixture by mass spectrometry provided a peak at 1055, which suggested Et$_3$N promoted β-elimination of one of the cyanoethyl protecting groups 85 (Scheme 34).

![Scheme 34: Reagents and conditions: i) a) Carbonyl diimidazole, Et$_3$N, DMF, 3 h; b) CH$_3$OH, 10 mins; ii) 74, DMF, 18 h.](image)

4.2.3 Pyrophosphate purification

As ion exchange HPLC analysis showed distinct peaks, initial purification was carried out on the same quaternary ammonium AG-MP1 resin (Bio-Rad). However, eluting with a gradient of 150 mM aq. TFA apparently caused the gradual cleavage of the 3'-TIPDS group and the only material obtained from this column was disappointing quantities of the desilylated compound 86. More controlled TIPDS deprotection was attempted by reacting the crude pyrophosphate 85 with a 10 % aqueous solution of TFA. This was monitored by reverse phase TLC and once complete, neutralised with 1 M NaOH and purified on a column of AG-MP1 resin, to afford a 10 % yield (from adenosine analogue 74) of pyrophosphate 86 (Scheme 35). This was a dissatisfying yield as the HPLC analysis and $^{31}$P NMR spectrum both showed a high conversion to the pyrophosphate.
Scheme 35: Reagents and conditions: i) 10% aq. TFA, 2 h.

It was suspected that residual sodium trifluoroacetate from the neutralisation increased the conductivity of the solution causing poor adhesion of the compound to the resin. Analysis showed that a 1% solution of 150 mM aq. TFA possessed a conductivity greater than 400 µS; therefore, to ensure correct adhesion of the products to the stationary phase a conductivity of less than 400 µS was required for loading. Unfortunately, this did not have a dramatic effect on the yield, therefore it was necessary to find a better purification technique.

Size exclusion chromatography was investigated as the product had a molecular weight larger than the two starting materials. Crude pyrophosphate mixture was applied to Biogel P2 beads (1.5 x 70 cm) and eluted using 250 mM NH₄OAc, but this failed to provide any separation. Reverse phase column chromatography was also explored, but co-elution of product and starting material was repeatedly observed. Ion exchange chromatography still offered the best separation due to the negatively charged product, so a different quaternary ammonium resin was sought that avoided the use of TFA.

Q-Sepharose ion exchange resin offers similar characteristics to AG-MP1, and was identified as a purification method incorporating a quaternary ammonium stationary phase. Instead of using 150 mM aq. TFA, this resin required 1 M triethylammonium bicarbonate (TEAB) (pH 7) as the counter ion. The conductivity of the solution still needed to be adjusted to less than 400 µS, but once loaded and eluted, this method produced pure pyrophosphate 85 as the triethylammonium salt, in 63% yield from starting adenosine 74, with the silyl ether still in place (Scheme 34). Excess Et₃N
needed to be removed, but this was accomplished with repeated co-evaporation with CH$_3$OH. Isolation and characterisation of this material confirmed that the removal of one cyanoethyl group had indeed occurred, with four cyanoethyl protons being observed in the $^1$H NMR spectrum, instead of eight (Figure 12).

![Figure 12: $^1$H NMR spectrum showing the signals for the residual cyanoethyl protecting group on 85.](image)

**4.2.4 Protecting group removal**

The base labile cyanoethyl and fluoride labile TIPDS protection were selected because of their potential to undergo global deprotection with the addition of tetrabutyl-ammonium fluoride (TBAF). Initial cleavage conditions were evaluated using the protected diphosphate adenosine precursor 74. After three hours treatment with 1 M TBAF in THF buffered with AcOH, an increase in the HPLC retention time was observed from 13 to 15 minutes. Purification on Q-Sepharose resin and $^1$H and $^{31}$P NMR spectroscopy of the isolated material revealed that both the cyanoethyl group and silyl protecting group had indeed been cleaved, but the 2' phosphate had undergone cyclisation to afford 2',3'-cyclic phosphate 87 (Scheme 36) characterised by $^{31}$P NMR spectroscopy (δ 20 ppm). This was confirmed in the $^1$H NMR spectrum by a downfield shift for the 3'-proton to 5.04 ppm together with an increased complexity due to phosphorus coupling.
Scheme 36: Reagents and conditions: i) 1 M TBAF, AcOH, THF, 0 °C.

It was suspected that cyclisation was the result of Et₃N catalysed attack on the free 2'-phosphate by the deprotected 3'-hydroxyl, during TEAB evaporation. While, deprotection appeared possible under these conditions, purification would need to be carried out using acidic AG-MP1. Repeating these reaction conditions with the protected pyrophosphate 85 afforded TIPDS cleavage, but removal the second cyanoethyl group did not occur leading to monoprotected NADP 86 in 75 % yield (Scheme 37). It may be that the deprotection observed in the model was due to nucleophilic substitution by the 3'-hydroxyl to afford the cyclic phosphate 87. To enhance the reaction the pH of the solution was increased by the removal of the acetic acid. As there was no longer a buffer present, any hydroxide ions formed by water contamination reacting with fluoride ions, would accelerate the β-elimination. Upon reaction with 85, phosphate deprotection was still elusive, with only the desilylated material 86 produced. However, this revealed the glycosidic linkage had a greater stability than previously expected.

Scheme 37: Reagents and conditions: i) 1 M TBAF, AcOH, THF, 0 °C.

Even though the global deprotection failed, it was satisfying to achieve the NADP 86 precursor in high yield that enabled further exploration into the removal of the final
protecting group. It was speculated that the negative oxygen on the mono-deprotected 2' phosphate repelled the TBAF, thus hampering β elimination (Scheme 38).

![Scheme 38: The repulsion of the negative oxygen and fluoride ion.](image)

Different methods were sought to try to remove the cyanoethyl group using stronger basic conditions. A number of literature methods including methanolic ammonia, tBuNH₂, Me₂NH and diisopropylethylamine were attempted but were all unsuccessful. All of these conditions only returned starting material, which although unsuccessful, indicated the pyridinium bond to be more stable than initially expected.

Methods were then sought to chemically neutralise the negatively charged oxygen atom to enable base cleavage of the remaining 2'-phosphate protecting group. Previous work during the synthesis of Calyculin A required the removal of bis-cyanoethyl protecting groups to unveil a free phosphate late in the synthesis. The first protecting group was removed under basic conditions by the addition of DBU, but difficulties in removing the second protecting group were observed. This problem was solved by quenching the newly generated negative charge by silylation of the oxygen using TMSCl. Complete deprotection of the second protecting group was achieved in-situ with DBU, allowing the formation of the free phosphate in excellent yield. However, no reference was made to the deprotection of the TMS groups. A further development used TMSCl with tetramethylguanidine (TMG) as base, to perform the β-elimination of the cyanoethyl groups. Methanolic ammonia was then added before the TMS groups were cleaved using mild hydrolysis with AcOH and water to afford the free phosphate (Scheme 39).
Scheme 39: i) TMS protection; ii) protecting group cleavage; iii) TMS cleavage.

TMSCl and TMG were explored in a trial reaction using fully protected 2',5'-adenosine bisphosphate 74. The methanolic ammonia step was removed, due to the potential hydrolysis of the pyridinium. The acid hydrolysis was also replaced by TBAF, with the aim of simultaneously cleaving both fluoride labile silicon groups (Scheme 40).

Scheme 40: Reagents and conditions: i) a) TMSCl, TMG, CH$_3$CN, 16 h; b) 1 M TBAF, THF, 3 h, 0 °C.

Unfortunately, there were difficulties in observing even monocyanoethyl cleavage by HPLC analysis and isolation of any materials proved impossible. Due to poor reactivity an alternative TMS donor was used instead TMSCl. Again, a trial reaction was undertaken using 2'-protected bisphosphate adenosine 74 to initially cleave the first cyanoethyl group, followed by in-situ TMS protection using $N,O$-bis(trimethylsilyl) acetamide (BSA). Subsequent cleavage of the second cyanoethyl with DBU and simultaneous silyl deprotection with TBAF generated a new peak by HPLC analysis at 14 minutes. Purification on AG-MP1 afforded 2',5'-ADP 87 in an unoptimised, 36% yield, as confirmed by $^1$H and $^{31}$P NMR spectroscopy (Scheme 41).
This method was transferred directly to the 2',3'-protected pyrophosphate 85, and the reaction monitored by HPLC. It was expected that the retention time of the product would be similar at 11 minutes, as the overall charge on the molecule should remain the same. This is because the $pK_a$ of the second phosphate hydroxyl is around 8-9 so only one 2' phosphate hydroxyl would be ionised during analysis. Upon addition of the BSA / DBU there was no visible change, but after treatment with TBAF the retention time surprisingly increased to 14 minutes. Purification on ion exchange chromatography afforded only the hydrolysed 2',5'-ADP 87 (Scheme 42).

It was suspected that silylation not only occurred at the 2'-phosphate, but also on the pyrophosphate backbone (Scheme 42) and once silylated, the pyrophosphate underwent hydrolysis. Milder conditions were therefore sought to cleave the TMS groups in the hope of avoiding pyrophosphate hydrolysis. TBAF was therefore replaced with an aqueous solution of TFA or AcOH, but both of these afforded 2',5'-ADP 87. Material, pre-treated with TBAF to cleave the monoprotected NADP 86, was subjected to BSA / DBU treatment, followed by, overnight stirring in H$_2$O to
hydrolyse the TMS groups. This did provide a poor yield, of a product that closely resembled NADP 7, but careful examination using mass spectrometry revealed a discrete peak at 765, possibly corresponding to the single sodium salt of NADP 7. Unfortunately, the $^1$H and $^{13}$C NMR spectra provided conflicting results, as no signal was detected for $2_N$H and $6_\Lambda$C. When this reaction was repeated, the de-purinated product was isolated, as characterised by the absence of the two purine protons in the $^1$H NMR spectrum. Further work will be required to understand what has taken place, especially as the changes observed relate to different parts of the molecule.

As treatment with BSA / DBU resulted in the total deprotection of the 2'-phosphate, a further method was sought to cleave the TMS groups, whilst avoiding pyrophosphate hydrolysis. Literature TMS deprotection has been performed using HF as the source of fluoride. It was hoped that the 3'-TIPDS along with all the TMS groups could finally be simultaneously removed with anhydrous acidic HF / pyridine to afford NADP 7. Purification of the result of this reaction on AG-MP1 resin unfortunately only provided nicotinamide adenine dinucleotide (2'-cyanoethoxy) phosphate 86. It remains unclear why the second cyanoethyl group remained intact as the initial reaction conditions should have removed the protecting groups and reactions reported in the literature using these conditions observe high yielding cleavage.
4.3 Summary

Scheme 43: Overview of the synthetic route adopted in this synthesis

This synthesis provided a high yielding route to the selectively phosphorylated adenosine (Scheme 43) and retains the flexibility to enable rapid analogue synthesis. Pyrophosphate bond formation was successfully optimised and two purification methods were developed, which ultimately provided high yields of pure pyrophosphate. Removal of the final protecting group to afford NADP 7 has proven elusive, but the chemistry developed for the removal of TIPDS group and knowledge gained from the TMS installation will prove invaluable in the development of a secondary route involving different protecting groups at the 2′-phosphate.
Chapter Five

Synthesis of NADP and NAADP
5.1 Introduction

In chapter four it was shown that protection of the 3'-and 5'-hydroxyls allowed installation of a phosphate group at the 2'-hydroxyl without spurious phosphorylation on the purinyl amine. Deprotection and subsequent phosphorylation of the 5'-hydroxyl led to the adenosine diphosphate intermediate 74. Pyrophosphate chemistry was extensively explored and the resultant CDI coupling proved very reliable affording high yields. Purification was aided by the serendipitous discovery of the cleavage of one phosphate protecting group, enabling isolation and characterisation. Subsequent deprotection however remained elusive and to this end restarting the synthesis with alternative 2'-phosphate protecting groups that could be cleaved under neutral conditions would be required for a route to synthetic NADP 7 and NAADP 3.

5.2 Selective 2', 5' phosphorylation of adenosine

It was anticipated that benzyl phosphoramidite 52, previously installed at the 5'-alcohol, could be added to the 2'-hydroxyl, ultimately enabling facile cleavage by hydrogenation in the latter stages of the synthesis. It was also expected to be stable to mild basic and acidic conditions and therefore, remain intact throughout the synthesis.

\[
\text{Scheme 44: Reagents and conditions: i) a) } \text{Bis(benzyl)diisopropylaminophosphine 52, imidazolium triflate 51, CH}_2\text{Cl}_2, 18 \text{ h; b) mCPBA, -78 °C, 30 mins.}
\]

The 3'-and 5'-positions were protected as previously described (see chapter four). Activation of the phosphitylating reagent required \textit{in-situ} reaction between bisbenzylphosphoramidate 52 with imidazolium triflate 51 to afford the phosphite at the 2'-hydroxyl. Subsequent oxidation with mCPBA afforded the desired phosphate 93 in 78 % yield after silica flash chromatography (Scheme 44). Regio-selective ring opening of the less hindered 5'-silyl-ether was accomplished using mild acidic conditions (TFA–H$_2$O–THF (1:1:4; v/v/v)) leading to the alcohol 94 in 76 % yield (Scheme 45).
A range of phosphate protecting groups were explored to generate the 5'-phosphate without degradation of the rest of the molecule. Treatment of 5'-alcohol 94 with bis(2-cyanoethyl)phosphoramidite 69 afforded diphosphate 95. It was hoped that strong basic conditions could be utilised to afford the free phosphate 96, however ten equivalents of LiOH destroyed the molecule after one hour with no recognisable peaks remaining in the $^1$H NMR spectrum (Scheme 46). Removal of the protecting groups was abandoned due to the problems associated with the release of the free phosphate. Only after this work was complete were conditions developed to remove both protecting groups effectively. A second problem was the possibility of base-catalysed cleavage of a benzyl group, which may have been reactive during pyrophosphate bond formation.

Exploring further possible protecting groups suggested that the allyl phosphate could be cleaved using mild, neutral conditions such as, Pd(PPh$_3$)$_4$, or Rh(PPh$_3$)$_3$Cl, that should be compatible with the rest of the adenosine functionality. Phosphoramidate chemistry with bisallyldiisopropyl-aminophosphate 69, imidazolium triflate 51, CH$_2$Cl$_2$, 5 h; b) mCPBA, -78 °C, 40 mins; ii) 10 equivalents LiOH, 1 h.

Exploring further possible protecting groups suggested that the allyl phosphate could be cleaved using mild, neutral conditions such as, Pd(PPh$_3$)$_4$, or Rh(PPh$_3$)$_3$Cl, that should be compatible with the rest of the adenosine functionality. Phosphoramidate chemistry with bisallyldiisopropyl-aminophosphate 69, imidazolium triflate 51, CH$_2$Cl$_2$, 5 h; b) mCPBA, -78 °C, 40 mins; ii) 10 equivalents LiOH, 1 h.
suggesting that a second protected phosphate had been installed (Scheme 47). This was further confirmed by mass spectrometry data, which showed a peak at 1108 consistent with either the formation of compound 97 or 98. Based on literature precedent we expected that phosphitylation would only proceed at the purinyl NH$_2$ if the more reactive phosphotetrazolide was used instead of the imidazolide. To date we have not been able to confirm whether the extra phosphate was bound to the purinyl NH$_2$ 97 or the silanol hydroxyl 98. However, an experiment carried out by Christelle Moreau using the protected purinyl amine still observed secondary phosphitylation, suggesting formation of the silanol phosphate related to structure 98. How an allyl-protected phosphoramidate is sufficiently reactive to attack either the deactivated amine or silanol hydroxyl remains unclear, especially when other phosphitylating reagents, such as $\textit{bis}(2$-cyanoethyl$)$-phosphoramidate 69, only reacted with the riboside hydroxyls.

Scheme 47: Reagents and conditions: i) a) $\textit{bis}$($\text{allyl}_{2}$-$\text{diisopropylaminophosphine}$, imidazolium triflate, CH$_2$Cl$_2$, 4 h; b) mCPBA, -78 °C, 40 mins.

A diverse range of phosphitylating reagents were screened within the group including the paramethoxybenzyl, tertiarybutyl and 2-(methylsulphonyl)ethyl protected phosphoramidites. Unfortunately these were unsuccessful due to difficulties in installing the phosphate using either the paramethoxybenzyl or tertiarybutyl phosphoramidites (102 and 101); however installation of the sulphonyl
phosphoramidite was possible, affording 99, but deprotection with 0.1 M NaOH destroyed the molecule (Scheme 48). It was suspected that the failure of the first two routes was due to steric hindrance around the primary hydroxyl. Interestingly though, no secondary phosphitylation was observed on either the purinyl amine or silanol hydroxyl even though large excesses were used to try and drive the reaction to completion.

Scheme 48: Overview of work exploring 5' phosphitylation.

The phosphoramidite chemistry was therefore abandoned and further methods of phosphate installation explored. Formation of H-phosphonates is a highly efficient reaction at primary, secondary and aromatic hydroxyls. The resulting H-phosphonate is poorly reactive and required activation prior to modification. Work by Christelle Moreau formed the triimidazolylphosphine by the \textit{in-situ} reaction between PCI\textsubscript{3} and imidazole. This species is a mild, yet highly reactive intermediate that upon treatment with hydroxyl efficiently formed the desired 5'-H-phosphonate. After quenching with 1 M aq. TEAB and silica flash chromatography the H-phosphonate 103 was isolated in 70 % yield (Scheme 49). The identity of this product 103 was confirmed by characteristic signals of the H-phosphonate\textsuperscript{69} in the \textsuperscript{31}P and \textsuperscript{1}H NMR spectra (\(\delta_p\) 6.8 ppm; \(\delta_H\) 6.72, \(^1J_{P,H}\) 630 Hz). The highly reactive P\textsuperscript{III} triimidazolylphosphine is also relatively small and may be able to overcome the steric hindrance difficulties encountered with the phosphoramidite chemistry. Quenching of
the reaction with triethylammonium carbonate led to the formation of an organic soluble salt that could be readily purified using silica flash chromatography.

**Scheme 49: Reagents and conditions:**

i) a) Imidazole, PCl$_3$, Et$_3$N, THF, 0 °C, 15 mins; b) 94, THF, 15 mins; c) 1 M aq. TEAB, 15 mins, ii) a) BSA, CDCl$_3$, 1 h; b) CSO, 15 mins; c) CH$_3$OH:CHCl$_3$:D$_2$O, 2 h.

Oxidation of the $H$-phosphonate is often difficult, but we chose to explore a recently described protocol. Treatment of the $H$-phosphonate with the silylating reagent bis(trimethylsilyl)acetamide (BSA) led to a highly reactive trivalent silyl phosphite 105, which can be oxidised to the silyl phosphate. It was reported that initial treatment with mCPBA failed to afford oxidised product, due to excess TMS silylating the peroxide. Oxidising agents were thus sought that did not possess acidic hydrogens. (1S)-(+)-(10-camphorsulphonyl) oxaziridine (CSO) 106 performs the oxidation in good yield with no observed side reactions. Consequently, reaction of $H$-phosphonate 103 with bis(trimethylsilyl)acetamide proceeded to completion in one hour (105). Subsequent oxidation using CSO afforded the bis(TMS) phosphate ester 107 in 15 minutes. The two TMS groups were then hydrolysed (with CH$_3$OH:CHCl$_3$:D$_2$O over two hours) to afford the free phosphate 104 in 50 % yield after flash chromatography (Scheme 49 and Scheme 50). This process can be conveniently monitored by $^{31}$P NMR spectroscopy (Figure 13) that demonstrates satisfyingly clean conversion between each reaction intermediate.

**Scheme 50: $H$-phosphonate oxidation to the phosphate with approximate NMR values and the structure of CSO Reagents and conditions:**

i) BSA, CDCl$_3$, 1 h; ii) CSO, 15 mins; iii) CH$_3$OH:CHCl$_3$:D$_2$O, 2 h.
5.3 Pyrophosphate bond formation

With the free phosphate generated, work could be carried out to synthesise the pyrophosphate with subsequent exploration of the deprotection strategy. Pyrophosphate synthesis was performed using the protocol established during previous studies. 58, 59 A solution of β-NMN 21 in DMF was activated with carbonyl diimidazole (CDI) and monitored by $^{31}$P NMR spectroscopy for generation of the

Figure 13: A $^{31}$P NMR comparison showing the chemical shift associated with the oxidation from $H$-phosphonate 103 to phosphate 104 of the 5'-alcohol. The stationary peak at δ -0.9 ppm is that of the 2'-benzyl phosphate.

It is interesting to note that the $H$-phosphonate is strongly UV active, however upon oxidation to the phosphate the chromophore is apparently quenched. This may be due to a change in conformation causing the aromatic rings on the protected phosphate to interact with the purine ring. This does however make visualisation of the subsequent reactions difficult as HPLC analysis requires a chromophore for detection.
phosphoimidazolide. CH$_3$OH was added to quench any remaining CDI, before the addition of the protected adenosine 104. The coupling was monitored by $^{31}$P NMR spectroscopy, which showed the disappearance of starting adenosine 104 after 24 hours and the appearance of the characteristic pyrophosphate multiplet at $\delta$ -10 ppm (Scheme 51).

Scheme 51: Reagents and conditions: i) a) Carbonyl diimidazole, Et$_3$N, DMF, 3 h then CH$_3$OH; b) 104, DMF, 24 h.

At this stage, HPLC analysis and purification of 108 proved difficult, as the product was still only slightly UV active. Both benzyl groups remained intact throughout the pyrophosphate formation resulting in an amphiphilic product that was difficult to purify by all chromatographic methods. Methods were therefore sought to cleave at least one benzyl group, generating a negative charge that could aid purification by ion exchange chromatography and hopefully enhance the UV activity of the molecule to enable detection.

5.4 Selective protecting group removal

A variety of hydrogenation conditions were explored using the crude pyrophosphate 108 to only release the 2'-phosphate with the aim of aiding purification. 10 % Pd/C with hydrogen and 10 % Pd/C with cyclohexadiene, failed to release the benzyl protected phosphate with only starting material recovered. Catalyst poisoning by the excess imidazole present from the coupling reaction may hinder this debenzylation. Attempts using 20 % Pd(OH)$_2$/C with cyclohexene at 80 °C resulted in the destruction of the material, presumably a result of the high temperature. Methods were therefore
sought to enable the cleavage of at least one benzyl group to allow purification on ion exchange.

A review of the literature showed that it was possible to achieve monobenzyl deprotection using either TBAF\textsuperscript{52} or aqueous TFA,\textsuperscript{71} which would be expected to proceed with concomitant desilylation (Scheme 52). The crude pyrophosphate product was treated with a 1:1 (v/v) mixture of TFA and H\textsubscript{2}O for one hour resulting in simultaneous monobenzyl deprotection and TIPDS cleavage. Monitoring by ion exchange HPLC showed a new peak at 11 minutes, with a retention time similar to peaks observed for the monocyaanoethyl pyrophosphate \textsuperscript{86}. Notably the monobenzyl NADP \textsuperscript{109} was strongly UV absorbing. Observation of the complete shift of the 2'-phosphate peak in the \textsuperscript{31}P NMR spectrum allowed monitoring of when the reaction was complete (δ\textsubscript{P} -1.0 ppm to -0.1 ppm). Complete TIPDS cleavage occurred, thus allowing isolation of the desired mono-protected benzyl NADP \textsuperscript{109} using AG-MP1 ion exchange resin, albeit, in a disappointing yield (<5% from starting adenosine \textsuperscript{104}).

Further crude pyrophosphate mixture \textsuperscript{108} was treated with 1 N TBAF in THF at 0 °C for two hours. AcOH was used to buffer the reaction to protect the labile pyridinium from base hydrolysis (Scheme 52). Again both the TIPDS and a single benzyl group were removed and the mono-benzyl protected NADP \textsuperscript{109} was produced in 14 % yield from adenosine 5' phosphate \textsuperscript{104} (Figure 14). Later experiments by Christelle Moreau marginally improved the overall yield to 23 %. Since the fully protected product \textsuperscript{108} could not be isolated, we could not conclude whether the pyrophosphate bond formation was high yielding, although we speculate that the main problem is the benzyl deprotection, based on results described in the previous chapter.
Scheme 52: Reagents and conditions: i) TFA:H₂O (1:1; v/v) 1 h, or 1 N TBAF, AcOH, THF, 0 °C, 2 h.

Figure 14: ¹H NMR spectrum showing the presence of the benzyl protection.

To check the debenzylation step, fully protected adenosine diphosphate 104 was treated with buffered TBAF, until TLC analysis (with staining) showed that all starting material had been consumed after two hours (Scheme 53). Analysis by ion exchange HPLC showed the desired peak at 14 minutes as well as many impurities. The desired bisphosphate 110 was obtained in only a 4 % yield. This is consistent with the destruction observed when the protected NADP 108 analogue was subject to the same conditions and justifies the idea that the poor yield can be attributed to this deprotection step. Further work would be required to explore any other methods for singular benzyl deprotection after pyrophosphate formation that may ultimately aid purification in the same way as for the monocyanoethyl protected NADP 85.
Scheme 53: Reagents and conditions: i) 1 M TBAF, AcOH, THF, 0 °C, 2 h.

It appears that to date, at least the best route to monobenzyl protected NADP 109 is using TBAF even though this has been shown to be destructive to the molecule with yields of around 20 %, however this could relate to two 50 % reactions if the pyrophosphate synthesis is not as efficient. It is important to note that the cyanoethyl protected compound afforded a 47 % yield (approximately two times 70 %) over the same two steps with visualisation and isolation available throughout the reactions.

5.5 Synthesis of NADP and NAADP

Isolation of pure monobenzyl NADP 109 did enable hydrogenation conditions to be explored to cleave the final benzyl group. 10 % Pd/C with hydrogen was dismissed as it was found to cleave the pyridinium base. An identical result was obtained when the same conditions were applied to commercial NADP. Transfer hydrogenation using 10 % Pd/C with cyclohexadiene in degassed solvents showed a downfield movement of the 2'-phosphate to δ 0.38 in the ³¹P NMR spectrum after two hours, indicating the formation of NADP 7. The mixture was passed through a membrane filter to remove the catalyst and the product eluted on a column of AG-MP1 resin to yield NADP 7 in an unoptimised 31 % (Scheme 54 and Figure 15).
Scheme 54: Reagents and conditions: i) 10 % Pd/C, cyclohexadiene, degassed CH₃OH:H₂O (1:3; v/v), 2 h.

Figure 15: ¹H NMR spectrum of NADP showing the total absence of benzyl protons.

Synthetic NADP 7 and nicotinic acid 22 were dissolved in 100 mM AcOH-NaOH buffered to pH 4 to which was added Aplysia ADP ribosyl cyclase [EC 3.2.2.5], used to catalyse the base-exchange reaction. The enzyme was used directly from crude extracts from the ovotestis of Aplysia californica⁷² as it was observed within the group that the quality of the commercially available enzyme varies greatly from batch to batch. The reaction was monitored by ion exchange HPLC for the characteristic increase in retention time, from 10 to 16 minutes (7 to 3; Figure 17), caused by the introduction of the acid functionality (Scheme 55). It can also be observed on the HPLC trace the formation of the side product of the reaction with is the hydrolysis of the pyridinium ring to form ADP-ribose (Rₚ 19 mins). Purification of the reaction by AG-MP1 ion exchange chromatography and isolation of the desired peak at 16 minutes afforded NAADP 3 in 60 % yield (Figure 16 and Figure 17).
Scheme 55: Reagents and conditions: i) Aplysia ADP ribosyl cyclase, nicotinic acid, 100 mM AcOH-NaOH (pH 4).

Figure 16: $^1$H NMR spectrum of NAADP 3.

Figure 17: HPLC trace (method A – see experimental general methods) of the enzymatic base-exchange reaction from NADP 7 to NAADP 3. Part A – Reaction after 1 h. B – Reaction after 3 h. C – reaction after 6 h Also shows the appearance of ADP-ribose as a side product. D – Purified NAADP.
5.6 Biological testing

NAADP 3 was quantified using the Brigg's test\textsuperscript{65} (see experimental general method) before being evaluated for inducing calcium release in sea urchin eggs. As the NAADP 3 had been produced from synthetic NADP 7 it was important to establish that its activity was identical to authentic NAADP (produced from the base-exchange reaction carried out on commercial NADP).

Biological results are obtained from homogenised sea urchin egg cells, diluted to 5\% and incubated at 17°C. A fluorescent dye, Fluo-3 is added to the suspension before the addition of NAADP. As calcium is released from the homogenate it binds to Fluo-3, causing it to fluoresce, which is detected and recorded. The organelles in the homogenates then pump down the free calcium in an ATP dependant manner, causing a gradual decrease in fluorescence. The cells can then be subjected to repeat injection by NAADP, but this time no calcium is observed as a result of its self-inactivation properties.

Synthetic NAADP 3 produces an identical calcium release profile to authentic NAADP and also produces the same self-inactivation profile when a subsequent batch is re-injected (Figure 18). It is also possible to competitively bind non-labelled, \textit{synthetic} NAADP 3 in preference to [\textsuperscript{32}P] NAADP in exactly the same concentration as non-labelled \textit{authentic} NAADP during binding experiments (Figure 19). This shows that the synthetic NAADP 3 is binding in the same way as authentic NAADP.
Figure 18: Representation of the calcium release profile observed when synthetic NAADP is compared to authentic NAADP.

Synthetic vs Authentic NAADP

Figure 19: Representation of the competitive binding of 0.2 nM $[^{32}\text{P}]$ NAADP with either synthetic or authentic NAADP.
5.6 Summary
Installing a phosphate on the 2'-hydroxyl with protecting groups that are labile to hydrogenation allowed the first total chemical synthesis of NADP 7 to be accomplished. Care was required to isolate the important monobenzyl intermediate 109 ensuring the least amount of material was degraded. Conversion to NAADP 3 afforded the first chemical synthesis to date of this novel calcium releasing second messenger. The biological analysis obtained has proven the identity of the material.

5.7 Future work
Further work will need to be undertaken to synthesise the nicotinic acid ribonucleotide to enable the total chemical synthesis of NAADP 3, but the overall route is a significant and essential step towards the development of analogues, such as C-glycosides that are inaccessible by any other means. This will allow the interrogation of this important biological pathway to further understand the importance of NAADP at a molecular level.

With a synthetic route in place, work can be undertaken to prepare analogues based on the core structure of NAADP. Initially, this will centre on the replacement of the pyridinium ring with a non-hydrolysable C-C bond to the ribose backbone to further understand whether the positive charge, or the labile bond are important in the mechanism of action.
Chapter Six

Synthesis of an NAADP analogue
6.1 Synthesis of a non-hydrolysable β-NaMN analogue

6.1.1 Introduction

With a synthetic route to NA(A)DP 7(3) developed\(^7^3\), exploration of analogues was pursued.

Previous reports have shown that replacement of the pyridinium ring with a 4-amide substituted thiazole, have inhibited some NAD dependent enzymes through its increased stability to hydrolysis.\(^3^4\) Tiazofurin\(^7^4\) **111** (2-(β-D-ribofuranosyl) thiazole-4-carboxamide) is a non-hydrolysable C-nucleoside analogue, which has known biological activity against both human lymphoid and lung tumour cells by being phosphorylated and linked to AMP via NMNAT \([E.C. \, 2.7.7.1]\) to afford thiazole-4-carboxamide adenine dinucleotide (TAD) **112** *in-vivo*.\(^3^4,75\) As TAD **112**, it mimics the structure of NAD \(^6\) (Figure 20) and inhibits inosine monophosphosphate dehydrogenase (IMPDH) \([EC \, 1.1.1.205]\) causing the suppression of *de-novo* biosynthesis of guanidine nucleotides.\(^3^4,75\) Subsequent decrease in guanidine monophosphate (GMP) and 2′-deoxyGMP not only hinders RNA and DNA synthesis, but also compromises the ability of G-proteins to function as transducers of intracellular signals. Tiazofurin **111** has been shown to be active in reducing the numbers of leukaemic cells in acute myelogenous leukaemia, however, it has been found to be too toxic for clinical application. Recent discovery of two different isoforms of IMPDH,\(^7^6\) which the type two is up-regulated in human leukaemia has promoted further interest in tiazofurin **111**.

We were interested in producing a carboxylic acid analogue of tiazofurin **113**, which when joined with an appropriately functionalised adenosine would lead to TAADP, **114** that would retain the non-hydrolysable C-C bond to the ribose backbone and also its aromaticity. It is anticipated that the negative charge would be held in a spatially similar position when linked to 2′,5′-ADP \(^8^7\). This could produce a novel NAADP analogue that may possess activity and enable further investigation of the mechanism of action of NAADP \(^3\) at a molecular level (Figure 20).
Figure 20: Schematic to show how tiazofurin (red) and its acid counterpart (blue) reflect the structure of the intended pyridinium.

Early syntheses towards tiazofurin 111 used H$_2$S to generate a thioamide *en route* to the thiazole ring, but H$_2$S is too toxic and difficult to work with on a large scale. A later synthesis described the formation of the thiazoline ring 115 from the β-cyano ribose 116 using cysteine ethyl ester. Despite testing a wide range of reactions the authors could not oxidise the thiazoline ring instead observing formation of the 1,4-substituted furan 117 (Scheme 56). To alleviate this problem, extra protecting group manipulations were introduced into the synthesis. The 2'-and 3'-benzoate esters were replaced with an isopropylidene group 118, thus eliminating the unnecessary ribose oxidation. This then allowed successful production of the thiazole 119 via the thiazoline 120, followed by subsequent two-stage deprotection to afford tiazofurin 111 (Scheme 56).
Scheme 56: Reagents and conditions: i) Cysteine ethyl ester, Et$_3$N, CH$_3$OH, 3 h; ii) MnO$_2$, benzene or N-bromosuccinimide, DBU; iii) cysteine methyl ester, Et$_3$N, CH$_2$Cl$_2$, 24 h; iv) MnO$_2$, benzene, reflux, 2 h; v) 90% TFA, 1 h; vi) methanolic ammonia, 12 h.

6.1.2 Synthesis of Tiazofurin and analogues

We believed that it was possible to perform the oxidation to the thiazole without concomitant elimination of the two benzoate esters. Commercially available tetra-ester protected ribose was treated with trimethylsilyl cyanide (TMSCN) and 1 M SnCl$_4$ in CH$_2$Cl$_2$, leading to the nitrile, in high yield as an orange foam (Scheme 57). The β-stereochemistry was introduced at this stage via neighbouring group participation and was confirmed by analysis of the anomic proton coupling constant of 4.4 Hz in the $^1$H NMR spectrum. Near quantitative conversion to the nitrile allowed the product to be used without chromatography of the crude material. Subsequent reaction with cysteine methyl ester led to the ring closure and the thiazoline, again with high conversion. Formation of the thiazoline ring was confirmed by the downfield shift in $^1$H NMR spectrum of the methylene and methine protons originally from the starting amino acid. Splitting of the signals was observed due to the epimerisation of the C-4 on the thiazoline ring. Resolution was not required however, as oxidation to the thiazole would remove the stereogenic centre.
Scheme 57: Reagents and conditions: i) TMSCN, 1 M SnCl4, CH2Cl2, 2 mins; ii) cysteine methyl ester, Et3N, CH2Cl2, 3 h; iii) BrCCl3, DBU, CH2Cl2, 12 h, 0 °C.

The thiazoline 123 was reacted with either I2–DBU or DDQ–toluene to perform the oxidation to thiazole 121, but these were found unsuccessful due side reactions complicating isolation.75 Milder BrCCl3–DBU79 treatment with the thiazoline 123, led to smooth dehydrogenation affording the thiazole 121 with no observed 2′,3′-benzoate elimination (Scheme 57). 1H NMR spectroscopy confirmed the oxidation by the presence of a single aromatic proton (δ 8.15), and the absence of thiazoline methylene and methine protons. The oxidation also removed the chiral centre and simplification of the spectrum was observed. Over the three steps (122 to 121) the isolated yield was 61 % (85 % per reaction), which was only subject to flash chromatography at this final stage, providing a high yielding, synthetic route.

Catalytic CH3ONa+ in CH3OH was utilised to afford the benzoate deprotection with retention of ester functionality to afford the methyl ester 124 as a white gum in 80 % yield. The tribenzoate precursor 121 was also treated with either methanolic ammonia, or 1 M NaOH to afford either tiazofurin 111 in 86 % yield, or the carboxylic acid analogue 113 in 86 % yield (Scheme 58).
Scheme 58: Reagents and conditions: i) CH₃ONa⁺, CH₃OH, 12 h; ii) 1 M NaOH, dioxane, 12 h; iii) methanolic ammonia, 36 h.

This route provided an improved synthesis of tiazofurin 111 and its analogues (124, 113) without the need for toxic H₂S and unnecessary protecting group manipulation. This route confirmed that the selective oxidation of a thiazoline in the presence of sensitive functionality, such as esters is possible with BrCCl₃ and DBU. To reach the end products required two chromatographic purifications for the methyl ester 124 or in the case of the carboxylic acid 113 and amide 111 only one due to their hydrophilic nature, making this a very fast and efficient route. Both amide 111 and the ester 124 could be used once phosphorylated, in the formation of a pyrophosphate bond to a suitably modified adenosine analogue that once deprotected would afford either a NADP⁻⁷ or NAADP⁻³ analogue respectively.

6.1.3 Phosphorylation of the primary hydroxyl

Phosphorylation was performed on the methyl ester 124 as it was suspected that the free carboxylic acid 113 might undergo side reactions during pyrophosphate formation. It was reasoned that the carboxylic acid could be unveiled by late stage treatment with alkali, such as 1 M NaOH, but that adequate protection on the 3'-adenosine hydroxyl may be necessary to avoid base catalysed 2'-phosphate cyclisation 125 and/or migration to the 3'-position 126 (Scheme 59).
Scheme 59: Reagents and conditions: i) 1 M NaOH.

Treatment of the ester 124 with triethylphosphate and POCI₃ resulted in the formation of a new peak by ion exchange HPLC analysis (Rₜ 14 minutes). As the product was negatively charged, purification by quaternary ammonium AG-MP1 ion exchange resin was possible. Isolation from the excess triethylphosphate afforded the phosphate 127 as a hygroscopic yellow gum in 72% yield after lyophilisation (Scheme 60). This material could then undergo pyrophosphate bond formation. Before that occurred, conditions were sought to provide ester hydrolysis that could be utilised later in the synthesis. Treatment with aqueous alkali (pH 10) produced an increase in retention time as observed by ion exchange HPLC, from 14 to 17 minutes indicating the formation of a second negative charge. Upon isolation using AG-MP1 resin, it was found that the ester 127 had indeed undergone hydrolysis to the acid 128. This was confirmed by the disappearance of the methyl peak in both the ¹H and ¹³C NMR spectra. This test reaction confirmed that late stage deprotection may be possible, thus allowing the phosphorylated methyl ester to be carried forward into the pyrophosphate synthesis.

Scheme 60: Reagents and conditions: i) Triethylphosphate, POCI₃, 0 °C, 90 mins; ii) H₂O (pH 10 with 1 M NaOH), 18 h.
6.2 Synthesis of the NAADP analogue

6.2.1 Pyrophosphate bond formation

The phosphorylated methyl ester 127 was subjected to pyrophosphate coupling using carbonyl diimidazole (CDI) through the formation of the activated phospho-imidazolide via the mixed anhydride. $^{31}$P NMR spectroscopy was used to monitor the reaction for the formation of the imidazolide peak at $\delta \sim$9.5 ppm, which occurred, as expected after three hours; however, the formation of a second peak at $\delta \sim$20 ppm was also observed, which remains difficult to explain. After excess CDI was deactivated with CH$_3$OH 2'-benzyl-phosphate adenosine 104 was introduced, and the reaction monitored for the appearance of the characteristic pyrophosphate multiplet at $\delta \sim$10 ppm and the disappearance of the adenosine free phosphate peak at $\delta$ 1.2 ppm (Scheme 61).

![Scheme 61: Reagents and conditions: i) a) CDI, Et$_3$N, DMF, 3 h; b) CH$_3$OH, 10 mins; c) 104, DMF, 24 h.](image)

After 24 hours the reaction appeared to be complete, as observed by $^{31}$P NMR spectroscopy and initial purification was carried out on silica flash chromatography to try and isolate the pyrophosphate 129. The product was poorly UV absorbing, presumably due to the quenching of the adenosine chromophore such that TLC only showed the appearance of a new product after staining with PMA ($R_F$ 0.5). Isolation of the material was difficult as the product 129 and starting material 104 have similar $R_F$'s. This did, however, remove excess imidazole from the reaction, allowing hydrogenation to be explored as for the synthetic pathway to NADP 7. Crude
material 129, was subject to 10% Pd/C-catalysed hydrogen transfer conditions to unveil the 2'-phosphate 130 (Scheme 62) that would enable purification by ion exchange chromatography. Analysis by HPLC provided no detectable evidence that debenzylolation had occurred, however $^{31}$P NMR spectroscopy showed complete conversion to the phosphate ($^{31}$P δ 1.5 ppm). The material was applied to a Q-Sepharose column, but attempts to elute the product with 1M aq. TEAB failed to provide product. Without the positive charge normally associated with NA(A)DP 7(3) the affinity to the quaternary ammonium resin could be greater, and increasing the concentration of buffer to 2M, did result in compound elution. This material was still impure, however and more importantly, the yield was very low.

Scheme 62: Reagents and conditions: i) 10% Pd/C, cyclohexadiene, CH$_3$OH/H$_2$O (3:1; v/v), overnight.

In light of the purification difficulties it was decided that the cyanoethyl protected adenosine 74 would be utilised as it had already provided higher yields along with being easier to handle as a result of the labile 2' phosphate ester. It was speculated that due to the increased stability of the C-glycoside linkage, strongly basic conditions could be used to perform the removal of the second phosphate-protecting group. Also, absence of benzyl groups would preserve the chromophore, thus enabling easier detection.
Scheme 63: Reagents and conditions: i) a) Carbonyl diimidazole, Et₃N, DMF, 3 h; b) CH₃OH, 10 mins; c) 74, DMF, 24 h.

The methyl ester 127 was activated following the established protocol and after three hours the cyanoethyl protected adenosine phosphate 74 was added and the reaction stirred overnight. Monitoring of the reaction by ³¹P NMR spectroscopy showed a complex signal around δ -10 ppm, in conjunction with the consumption of the 5'-adenosine phosphate peak 74. Analysis by ion exchange HPLC showed the formation of a new peak at 18 minutes enabling the reaction to be purified on Q-Sepharose ion exchange resin and product eluted using 2 M aq. TEAB. HPLC analysis showed the product remained impure however, with a second peak at 14 minutes, that corresponded to starting material 74. Even though the ion exchange HPLC retention times are different for starting adenosine phosphate 74 and newly formed pyrophosphate 131, Q-Sepharose resin seemed unable to resolve them. At this stage it was not possible to use AG-MPI ion exchange resin, as TFA would cause the cleavage of the 3'-TIPDS protection, which is required until basic ester hydrolysis has occurred to ensure that 2'-phosphate migration does not occur (Scheme 59). It was therefore decided to use the product crude in the subsequent phosphate deprotection.

6.2.2 Protecting group cleavage

It was speculated that the base labile cyanoethyl group would undergo β-elimination using a strong base, such as aqueous NaOH. As no other base sensitive groups are contained in the molecule and the 2'-phosphate can not migrate, NaOH would afford the concomitant phosphate deprotection and thiazole-methyl ester cleavage to afford
the 3'-protected TAADP 132. After treatment of crude 131 with aqueous NaOH (pH 10) overnight the \(^{31}\text{P}\) NMR spectrum showed the downfield movement of the phosphate peak from \(\delta -2.0\) ppm to \(\delta 4.2\) ppm suggesting that cyanoethyl deprotection had proceeded as expected (Scheme 64). HPLC analysis showed a new peak at 20 minutes, a result of the carboxylic acid negative charge, but again purification on Q-Sepharose resin afforded a mixture of products, so the product was used crude in the subsequent TIPDS cleavage.

Finally, with the basic reactions accomplished the 3'-TIPDS\(^ {67}\) protection could be removed using acidic HF in pyridine for 20 minutes. Purification on AG-MP1 allowed successful separation of the two materials and isolation of TAADP 114 as a white hygroscopic powder. Unfortunately, when analysed this material was found to be contaminated with approximately 10 % of an unknown compound. It is speculated that this maybe a result of degradation producing 2',5'-ADP 87, but further analysis will be required to confirm this (Figure 21).

**Scheme 64: Reagents and conditions: i) H\(_2\)O (adjusted to pH 10 with 1 M NaOH), overnight; ii), HF / pyridine (7/3), pyridine, 20 mins.**

Figure 21: \(^1\text{H}\) NMR spectrum of TAADP 114.
6.3 Biological results

To enable accurate phosphate quantification a Briggs' test\textsuperscript{65} (see experimental general methods) was carried out on TAADP \textbf{114} before being evaluated for calcium release properties within sea urchin egg homogenates. Preliminary data suggest that crude TAADP \textbf{114} does cause calcium release from a pool receptive to either Ins(1,4,5)P\textsubscript{3} \textbf{1}, cADPR \textbf{2} or NAADP \textbf{3} as the data shows a depleted release in response to subsequent activation by TAADP \textbf{114} (Figure 23). It has been rationalised that the calcium store depleted by TAADP \textbf{114}, is that stimulated by Ins(1,4,5)P\textsubscript{3} \textbf{1} as successive exposure leads to a minimal response (Figure 24). Both cADPR \textbf{2} and NAADP \textbf{3} are unaffected by this store depletion and function as normal. Initial indication therefore is that TAADP \textbf{114} is an Ins(1,4,5)P\textsubscript{3}R partial agonist. This is a very interesting discovery for two reasons. Firstly it does in fact cause calcium release and secondly it appears to be from a store other than that of NAADP \textbf{3}. This was a surprising result, as the SAR data lead us to believe that TAADP \textbf{114} could be a potential NAADP receptor agonist, along with a longer duration of action if pyridinium hydrolysis was the mechanism of inactivation. TAADP \textbf{114} does share some similarity to the potent Ins(1,4,5)P\textsubscript{3} agonist adenophostin \textbf{134} insomuch as the adenosine is phosphorylated at the 2'-position, but this does not account for the thiazole component, but may explain some of the observed activity (Figure 22).

\begin{center}
\textbf{Figure 22: Adenophostin}
\end{center}

It has also been shown that stores inhibited by thapsigargin are not responsive to calcium release by TAADP \textbf{114} and that the release from NAADP stores is independent of TAADP \textbf{114} concentration, confirming its action on the Ins(1,4,5)P\textsubscript{3}R. At this stage though the material that is being tested does contain a small percentage of impurity as observed in Figure 21 and this does need to be characterised to enable accurate data to be obtained.
**Figure 23:** Data representing depletion of all three discrete calcium stores followed by subsequent exposure to TAADP 114 causing no calcium release.

**Figure 24:** Data representing the depletion of Ins(1,4,5)P$_3$ sensitive calcium stores after exposure to TAADP 114. Calcium stores responsive to cADPR 2 and NAADP 3 are unaffected.

### 6.4 Summary

This synthetic route provided an analogue, which has undergone biological testing. It brings together two very high yielding and efficient synthetic schemes through the formation of a pyrophosphate bond between phosphorylated thiazole riboside 127 and cyanoethyl protected adenosine bisphosphate 74. Unfortunately, even though the pyrophosphate formation appeared high yielding, problems with purification meant that the intermediates could not be accurately characterised. It has been possible though to synthesise an analogue that retains the 2'-phosphate, without formation of the 3'-regio-isomer, whilst retaining the negative charge on the aromatic ring.
Disappointingly, it proved problematic to remove the small percentage of impurity from this sample and to obtain indisputable biological results, requires this to be accomplished.

6.5 Future work

Isolation of the impurity observed in the sample of TAADP 114 is important and as it is currently suspected to be 2',5'-ADP 87, this could be synthesised by the hydrolysis of NADP 7. Work will need to be carried out to try and develop the purification techniques to allow the isolation of the intermediates from the pyrophosphate formation. This will not only allow accurate quantification and characterisation of the compounds, but will also help overall yield optimisation by highlighting the poor reactions. For the purposes of biological evaluation further analogues would need to be produced to provide standards and also SAR information. These would need to include the amide derivative of the carboxylic acid, TADP 135 and also the dephosphorylated product TAAD 136 (Scheme 65).

Scheme 65: Proposed analogues required for biological testing. The FGI to the amide, TADP 135 and the dephosphorylation to TAAD 136.
Experimentals

7.1 General methods

All compounds were used as received unless otherwise stated. $^1$H and $^{13}$C NMR spectra were recorded on either a Jeol JMX GX-270 MHz, Varian EX-400 MHz or 600 MHz Inova spectrometer using deuterated chloroform, acetone, methanol, dimethylsulfoxide or D$_2$O. $^{31}$P NMR reaction monitoring was accomplished in either a deuterated solvent or via an internal capillary filled with either CD$_3$COCD$_3$ or CD$_3$OD. $^{31}$P NMR chemical shifts were measured in ppm and relative to external reference 85 $\%$ H$_3$PO$_4$. The following abbreviations are used to describe the signals in the $^1$H NMR spectra. s, singlet; br, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet. For dinucleotides, the top half (e.g. nicotinamide ribose) is no prime and the bottom half (e.g. adenosine) is prime. To differentiate the base (e.g. adenine) is by subscript letters (e.g. ad). The ribose moiety is without letters.

Thin layer chromatography (TLC) was performed on precoated plates (Merck silica gel 60 F$_{254}$ aluminium sheets) with visualization using UV light and staining with KMnO$_4$, anisaldehyde in EtOH and phosphomolybdic acid in MeOH. Column chromatography was carried out using silica gel (Matrix 60 particle size 35 – 70 microns).

Mass spectrometry data were obtained using a Micromass Autospec machine. IR spectra were obtained using a Perkin Elmer spectrum RX IFT-IR using either a KBr disk or thin film and are expressed as cm$^{-1}$.

Optical rotations were obtained at ambient temperature using an Optical Activity Ltd AA-10 polarimeter in a cell volume of 1 ml or 5 mL and specific rotation are expressed as $^{\circ}$ ml g$^{-1}$ dm$^{-1}$.

Melting points were determined using a Reichert-Jung Therm Galen Kofler block and are uncorrected.

CH$_3$CN was distilled from CaH$_2$ and stored over 4 Å molecular sieves. Et$_3$N was distilled from Et$_3$N and stored over KOH. Pyridine was distilled from KOH and stored over KOH. Trifluoroacetic anhydride was freshly distilled from P$_2$O$_5$ and used immediately.

Ion exchange analysis was carried out on a Hewlet Packard 1050 HPLC using an AG MP-1 column (3 x 150 mm) with UV detection set at 254 nm. Small particles were removed from the AG MP-1 by suspending in a large volume of Milli-Q (MQ) water.
(100 mL / 10g) then discarding any material left in suspension. The AG MP-1 was washed with 1 M NaOH (20 mL / g) then with MQ water until pH < 9. This was then washed with 150 mM aq. trifluoroacetic acid (TFA) (20 mL / g). To pack the column, two columns were joined together with a sleeve, securing them vertically, fitting an end cap with a frit to the bottom, then adding the slurry and allowing it to settle by gravity. An end cap with a frit was fitted to the column and it was then attached to the HPLC machine and 150 mM aq. TFA was pumped through at 5 mL / min for 15 minutes to pack the column. The sleeve was then removed and the bottom column fitted with an end cap with a frit. The compounds were then eluted from the column using a gradient of water – 150 mM aq. TFA to elute the compounds.

Method A: 1 minute - 1% aq. TFA, 2 minutes - 2% aq. TFA, 5 minutes - 4% aq. TFA, 9 minutes - 8% aq. TFA, 13 minutes - 16% aq. TFA, 17 minutes - 32% aq. TFA, 18 minutes - 100% aq. TFA, 21.10 minutes - 0% aq. TFA, 25 minutes - 0% aq. TFA.

Reverse phase ion exchange chromatography was carried out on a Phenomenex Aqua column, 5 microns, C18; 125A 150 x 4.60 mm.

Method B: 100 % H2O for 10 mins, linear gradient to H2O:CH3CN 1:1 over 20 mins, linear gradient to 100 % H2O over 1 min, 100 % H2O for 5 mins.

Product isolation using ion exchange was carried out on a Pharmacia LKB-gradifac. The conductivity of solutions loaded onto the resins was adjusted to less than 400 μS. AG-MP1 resin was eluted using a linear gradient of water – 150 mM aq. TFA eluting at 2 mL / min and Q-Sepharose fast flow resin eluted using a linear gradient of either 1 or 2 M aq. TEAB at 5 mL / min. Both methods collected 10 mL fractions per test tube. Peak determination was carried out using UV absorption at 260 nm.

Synthetic phosphates were assayed by adaptations of the Brigg's test as follows. For a quantitative test a standard curve was generated to calculate the quantity of the synthetic compound. Care was taken to use clean test tubes free from detergent. Using a series of tubes (in triplicate) increasing quantities of 10 mM KH2PO4 (10 nmol phosphate in 10 μL) were added.
Tube 1 0 μL phosphate 200 μL MQ water.
Tube 2 10 μL phosphate 190 μL MQ water.
Tube 3 20 μL phosphate 180 μL MQ water.
Tube 4 30 μL phosphate 170 μL MQ water.
Tube 5 40 μL phosphate 160 μL MQ water.
Tube 6 50 μL phosphate 150 μL MQ water.
Tube 7 60 μL phosphate 140 μL MQ water.
Tube 8 30 μL of compound 170 μL MQ water.
Tube 9 40 μL of compound 160 μL MQ water.
Tube 10 50 μL of compound 150 μL MQ water.

Tubes were placed in the oven at 180 °C for 15-30 minutes to evaporate to dryness. Once dry 4 drops of conc. H₂SO₄ was added to each tube and replaced in the oven for 90 minutes at 180 °C.

The following solutions were then freshly prepared.

Solution 1 – ammonium molybdate (5 g) in MQ water (40 mL) and conc. H₂SO₄ (16 mL) while cooling.

Solution 2 – hydroquinone (200 mg) in MQ water (40 mL) and conc. H₂SO₄ (2 drops).

Solution 3 – sodium sulphite (8 g) in MQ water (40 mL).

Tubes were the removed and cooled to room temperature.

To each tube was added: 250 μL of MQ water
500 μL of solution 1
250 μL of solution 2
250 μL of solution 3

The resulting mixtures were heated until boiled for 10 seconds.

Each solution was diluted with MQ water to 10 mL in a volumetric flask.

Each sample was analysed using a UV spectrophotometer at 340 nm.

Quantification of the compound is then calculated using the standard curve.
Extraction of ADP-ribosyl cyclase enzyme from ovotestis.

Buffer: 250 mM sucrose, 20 mM HEPES pH 8, 1 mM EDTA, 1 mM phenylmethyl-sulfonylfluoride (PMSF) and 1 μg/mL leupeptin.

2.8 g of ovotestis were homogenised in buffer (5:1 buffer volume to wet weight of tissue) followed by a two-step centrifugation procedure. The first step was a low speed centrifugation (13000 rpm at 4 °C for 20 minutes). The pellets were then discarded and the supernatant was subjected to a high-speed centrifugation (37000 rpm at 4°C for 2 hours). The supernatant was collected and the enzyme activity was assayed by completing the synthesis of NAADP.

Enzyme assay

5 mM NADP (4 mg) and 100 mM nicorinic acid (12 mg) in a 100 mM solution of AcOH / NaOH (pH 4, 1 mL) were incubated with 5 μL of crude enzyme at room temperature. After 5 hours, HPLC analysis (method A) showed complete consumption of NADP and formation of NAADP ($R_T = 16$ minutes).
7.2 Synthesis of pyridinium ribonucleotides

1-Nicotinamide-β-D-ribose

Nicotinamide (86 mg, 0.70 mmol) and trimethylsilyl trifluoromethanesulphonate (TMSOTf) (1.0 mL, 5.2 mmol) were added to a solution of tetra-1,2,3,5-O-acetyl-β-D-ribose (200 mg, 0.63 mmol) in CH₃CN (5 mL) and stirred at room temperature under a nitrogen atmosphere for one hour after which time TLC (CH₃OH:CH₂Cl₂ 1:9; R_f 0.1) showed complete conversion to a new peak. The reaction was then treated with CH₃OH (2.5 mL) and stirred for a further thirty minutes. The reaction was then evaporated to dryness, suspended in H₂O (20 mL) and extracted three times with butan-1-ol (3 x 20 mL). The organic layer was extracted with H₂O (3 x 10 mL) and the combined aqueous layers washed five times with CH₂Cl₂ (5 x 10 mL). The aqueous layer was collected and evaporated to dryness. The residue was subjected to flash chromatography (CH₂Cl₂ to CH₂Cl₂:CH₃OH; 8:2) to afford the desired product 39 as a pale orange syrup (232 mg, 91%).

R_f 0.35 (butan-1-ol:AcOH:H₂O, 5:2:3); ¹H NMR δ_H (270 MHz; D₂O) 9.60 (1H, s, H-2_nic), 9.27 (1H, d, J 6.2, H-6_nic), 8.98 (1H, d, J 7.9, H-4_nic), 8.27 (1H, m, H-5_nic), 6.24 (1H, d, J 4.2, H-1′), 4.52-4.47 (2H, m, H-2′, H-3′), 4.35 (1H, m, H-4′), 4.05 (1H, dd, J 13.1, 2.7, H-5′_a) and 3.89 (1H, dd, J 13.1, 3.3, H-5′_b); ¹³C NMR δ_C (100 MHz; D₂O) 164.5 (C=O), 144.8, 141.6, 139.4 (CH), 133.1 (C), 127.8, 99.3, 87.1, 76.8, 69.3 (CH) and 59.9 (CH₂); m/z [FAB⁺] 255.1 (M⁺, 98 %) [found 255.0982 C₁₁H₁₅N₂O₅ requires 255.0981].
1-Methylnicotinate-β-D-ribose

Methyl nicotinate (96 mg, 0.70 mmol) and TMSOTf (1.0 mL, 5.2 mmol) were added to a solution of tetra-1,2,3,5-O-acetate-β-D-ribose (200 mg, 0.63 mmol) in CH$_3$CN (5 mL) and stirred at room temperature under a nitrogen atmosphere for one hour after which time TLC (CH$_3$OH:CH$_2$Cl$_2$ 1:9) showed complete conversion to a new peak (R$_f$ 0.15). The reaction was then treated with CH$_3$OH (2.5 mL) and allowed to stir for a further thirty minutes. The reaction was evaporated to dryness, suspended in H$_2$O (20 mL) and extracted with butan-1-ol (3 x 20 mL). The organic layer was back extracted three times with H$_2$O (3 x 10 mL) and the combined aqueous layers were washed five times with CH$_2$Cl$_2$ (5 x 10 mL). The aqueous layer was collected and evaporated to dryness. The residue was subjected to flash chromatography (CH$_2$Cl$_2$ to CH$_2$Cl$_2$:CH$_3$OH; 8:2) to produce the desired product 40 as an orange syrup (215 mg, 81%).

R$_f$ 0.38 (butan-1-ol:AcOH:H$_2$O, 5:2:3); $^1$H NMR δ$_H$ (270 MHz; CD$_3$OD) 9.81 (1H, s, H-2$_{nic}$), 9.40 (1H, d, J 6.3, H-6$_{nic}$), 9.85 (1H, d, J 7.9, H-4$_{nic}$), 8.27 (1H, dd, J 7.9, 6.3, H-5$_{nic}$), 6.18 (1H, d, J 4.4, H-1$'$), 4.44-4.38 (2H, m, H-2$'$, H-3$'$), 4.30 (1H, m, H-4$'$), 4.03 (3H, s, OCH$_3$), 3.98 (1H, m, H-5$_a$) and 3.84 (1H, dd, J 12.4, 2.5, H-5$_b$); $^{13}$C NMR δ$_C$ (100 MHz; CD$_3$OD) 162.1 (C=O), 146.9, 143.5, 142.0 (CH), 131.1 (C), 128.3, 101.2, 89.0, 78.4, 70.7 (CH), 60.7 (CH$_2$) and 53.2 (CH$_3$); m/z [FAB$^+$] 270.2 (M$^+$, 100 %) [found 270.0981 C$_{11}$H$_{15}$NO$_6$ requires 270.0978].
Benzyl nicotinate

Benzyl alcohol (0.76 mL, 7.39 mmol), DCC (1.7 g, 8.13 mmol) and DMAP (90 mg, 0.81 mmol) were added to a solution of nicotinic acid (1.0 g, 8.13 mmol) in CH₂Cl₂ (36 mL) under a nitrogen atmosphere. The solution was stirred at room temperature overnight after which the resulting precipitate was removed by filtration and washed with CH₂Cl₂. The organic phase was extracted with aq. saturated NaHCO₃ (3 x 50 mL) and brine (3 x 50 mL), dried (Na₂SO₄), filtered and evaporated to dryness under vacuum. The resulting oil was subject to flash chromatography on silica gel using a gradient of CH₂Cl₂ to CH₂Cl₂:CH₃OH (100 to 98:2) as eluent. The required fractions were collected and evaporated to dryness to afford the ester 44 as a clear colourless oil (1.0 g, 67%).

¹H NMR δH (400 MHz; CDCl₃) 9.21 (1H, s, H-2nic), 8.69 (1H, d, J 4.7, H-6nic), 8.24 (1H, d, J 7.8, H-4nic), 7.42-7.39 (2H, m, H-Ar), 7.37-7.28 (4H, m, H-5nic, H-Ar) and 5.35 (2H, s, CH₂); ¹³C NMR δC (100 MHz; CDCl₃) 165.1 (C=O), 153.6, 151.1, 151.0, 137.3 (CH), 135.7 (C), 128.9, 128.7, 128.5 (CH), 126.2 (C) and 67.4 (CH₂). 2 carbon signals equivalent; m/z [FAB⁺] 214.2 (M⁺, 100 %) [found 214.0865 C₁₃H₁₂NO₂⁺ requires 214.0868].

1-Benzynicotinate-β-D-ribose

Benzyl nicotinate 44 (147 mg, 0.69 mmol) and TMSOTf (1 mL, 5.2 mmol) were added to a solution of tetra-1,2,3,5-O-acetate-β-D-ribose (200 mg, 0.63 mmol) in CH₃CN (5 mL). This was stirred at room temperature under a nitrogen atmosphere
for two hours after which time TLC (CH$_2$Cl$_2$:CH$_3$OH 9:1) showed complete conversion to a new peak (R$_f$ 0.5). The reaction mixture was then treated with CH$_3$OH (2.5 mL) and allowed to stir for a further hour. The reaction was evaporated to dryness and the resulting oil subject to flash chromatography on silica gel using a gradient of CH$_2$Cl$_2$ to CH$_2$Cl$_2$:CH$_3$OH (10 to 8:2). The required fractions were collected and evaporated to dryness to afford the benzyl nicotinate ribose 45 as a pale brown oil (254 mg, 82%).

$\nu$$_{\text{max}}$ (NaCl disk)/cm 3401 (OH), 1651 (COOBn); $^1$H NMR $\delta$ (400 MHz; CD$_3$OD) 9.70 (1H, s, H-2$_{\text{nic}}$), 9.32 (1H, d, J 6.3, H-6$_{\text{nic}}$), 9.00 (1H, d, J 8.1, H-4$_{\text{nic}}$), 8.19 (1H, dd, J 8.1, 6.3, H-5$_{\text{nic}}$), 7.45-7.43 (2H, m, H-Ar), 7.34-7.25 (3H, m, H-Ar), 6.17 (1H, d, J 4.7, H-1'), 5.42 (2H, s, CH$_2$Ar), 4.44 (1H, t, J 4.7, H-2'), 4.38 (1H, dd, J 5.8, 2.5, H-4'), 4.31 (1H, m, H-3'), 3.95 (1H, dd, J 12.5, 2.5, H-5'$_a$) and 3.83 (1H, dd, J 12.5, 2.5, H-5'$_b$); $^{13}$C NMR $\delta$ (67.5 MHz; CD$_3$OD) 162.2 (C=O), 147.63, 147.61, 144.1, 142.7 (CH), 135.7, 131.5 (C), 129.2, 129.1, 129.0, 101.7, 89.6, 78.8, 71.2 (CH), 69.0, 61.2 (CH$_2$). 2 carbon signals equivalent; m/z [FAB$^+$] 346.1 (M$^+$, 100%).

1-Nicotinic acid-β-D-ribose

Repeat of method for 1-Benzynicotinate-β-D-ribose but after silica flash chromatography the product was then fractionated between EtOAc (20 mL) and water (20 mL). The organic layer was extracted with water (3 x 5 mL) and the aqueous layer extracted with EtOAc (3 x 20 mL). The combined aqueous layers were evaporated to dryness to generate the acid 41 as a pale yellow oil (220 mg, 87%).

$\nu$$_{\text{max}}$ (NaCl disk)/cm 3422 (OH), 1919 (COOH); $^1$H NMR $\delta$ (400 MHz; D$_2$O) 9.38 (1H, s, H-2$_{\text{nic}}$), 8.90 (1H, d, J 6.3, H-6$_{\text{nic}}$), 8.74 (1H, d, J 9.2, H-4$_{\text{nic}}$), 7.92 (1H, m, H-5$_{\text{nic}}$), 5.89 (1H, d, J 3.9, H-1'), 4.15 (1H, m, H-2'), 4.13 (1H, m, H-4'), 4.01 (1H, t, J 4.9, H-3'), 3.73 (1H, m, H-5'$_a$) and 3.55 (1H, dd, J 12.9, 3.5, H-5'$_b$); $^{13}$C NMR $\delta$ (67.5 MHz; D$_2$O) 162.3 (C=O), 147.3, 143.6, 142.3, 128.6 (CH), 122.8 (C), 101.3,
89.6, 78.6, 71.2 (CH) and 61.0 (CH₂); m/z [FAB⁺] 256.1 (M⁺, 100%) [found 256.0819 C₁₃H₁₄NO₅⁺ requires 256.0821].

β-Nicotinamide mononucleotide (β-NMN)³⁷

Nicotinamide ribose triflate 39 (350mg, 0.87 mmol) was treated with activated charcoal and freeze dried from H₂O. This was suspended in a solution of trimethylphosphate (4.5 mL) and water (12 µL, 0.67 mmol) at 0°C. POCl₃ (528 µL, 5.6 mmol) was added and the reaction stirred at 0°C for four hours after which time a second portion of POCl₃ (200 µL, 2.1 mmol) was added. After a further hour a solution of CH₃CN:ether (1:3) as added causing the formation of a white precipitate. The supernatant was removed, the precipitate dissolved in water (5 mL) and applied to a Dowex 1 X 2 formamide (1 cm x 5 cm), which was eluted with water (50 mL). The volume was reduced under vacuum to 5 mL and the solution applied to a second column of Dowex 50 WX8-100 H⁺ (1 cm x 5 cm) washed with water (50 mL). The solution was evaporated to dryness and lyophilised from D₂O to afford β-NMN 21 as a white solid (42 mg, 14%).

¹H NMR δH (400 MHz; D₂O) 9.23 (1H, s, H-2′nic), 9.04 (1H, d, J 6.3, H-6′nic), 8.75 (1H, d, J 7.8, H-4′nic), 8.07 (1H, dd, J 7.8, 6.3, H-5′nic), 6.00 (1H, d, J 5.4, H-1′), 4.43 (1H, m, H-2′), 4.33 (1H, m, H-3′), 4.22 (1H, m, H-4′), 4.14 (1H, ddd, J 12.1, 4.3, 2.3, H-5′a), 3.97 (1H, ddd, J 12.1, 5.0, 2.3, H-5′b); ¹³C NMR δC (100 MHz, D₂O) 165.7 (C=O), 145.8, 142.3, 139.8 (CH), 133.8 (C), 128.4, 99.8, 87.2, 77.6, 70.8 (CH) and 64.3 (CH₂); ³¹P NMR δP (109 MHz; D₂O) 0.96; m/z [FAB⁺] 334.2 (M⁺, 80%)
5-O-Triphenylmethyl-β-D-ribose

Triphenylchloromethane (5.4 g, 19.5 mmol) was added to a solution of β-D-ribose (3.0 g, 20.0 mmol) in pyridine (10 mL) and stirred at 45°C for sixty minutes. The solution was evaporated to dryness under vacuum, re-suspended in CH₂Cl₂ (20 mL) and extracted with H₂O (3 x 10 mL). The organic layer was dried (MgSO₄), filtered and evaporated to dryness. The residue was dissolved in CH₂Cl₂ (6 mL), added dropwise to a solution of hexane (20 mL) and CH₂Cl₂ (2 mL) and stirred at room temperature for one hour. The resulting precipitate was filtered, washed with hexane and H₂O and dried at 50°C under vacuum to afford the 5'-protected ribose 47 as a white solid (5.1 g, 67%).

mp 124-128°C (from hexane) (lit. 125°C); ¹H NMR δH (400 MHz; CDCl₃) 7.49-7.44 (6H, m, H-Ar), 7.32-7.22 (9H, m, H-Ar), 5.42 (1H, d, J 4.0, H-1), 4.81 (1H, br s, OH-1), 4.27 (1H, dd, J 7.0, 3.8, H-4), 4.23 (1H, m, H-3), 4.07 (1H, m, H-2), 3.98 (1H, br s, OH-3), 3.58 (1H, br s, OH-2), 3.32 (1H, dd, J 10.1, 3.8, H-5a) and 3.13 (1H, dd, J 10.1, 3.8, H-5b); ¹³C NMR δC (100 MHz; CDCl₃) 143.7 (C), 128.7, 127.9, 127.2, 96.8 (CH), 86.9 (C), 83.2, 72.0, 71.9 (CH) and 64.1 (CH₂). 2 C and 14 CH equivalent.
1,2,3-Tri-O-acetyl-5-triphenylmethyl-β-D-ribose

Acetic anhydride (4.1 mL, 43.4 mmol) and Et₃N (6.9 mL, 50 mmol) were added to a solution of 5-trityl-β-D-ribose 47 (4.9 g, 12.5 mmol) in toluene (12.5 mL) and stirred at room temperature overnight. Iced water (15 mL) was added and the reaction stirred for a further ten minutes, separated and the aqueous layer extracted with toluene (3 x 10 mL). The combined organic layers were collected, washed with water (2 x 10 mL), dried (MgSO₄), filtered and evaporated to dryness. The oil was subject to flash chromatography on silica gel using a gradient of hexane to hexane-EtOAc (7:3) as the eluent to afford the triacetate protected ribose 48 as a white foam (4.43 g, 69%).

**H NMR** \( \delta_{\text{H}} \) (270 MHz; CDCl₃) 7.46-7.43 (6H, m, H-Ar), 7.31-7.22 (9H, m, H-Ar), 6.20 (1H, d, \( J = 1.0 \), H-1), 5.49 (1H, dd, \( J = 7.0, 4.8 \), H-3), 5.43 (1H, dd, \( J = 4.8, 1.0 \), H-2), 4.07 (1H, ddd, \( J = 7.0, 4.1, 3.5 \), H-4), 3.35 (1H, dd, \( J = 10.4, 3.5 \), H-5a), 3.14 (1H, dd, \( J = 10.4, 4.1 \), H-5b), 2.03 (3H, s, CH₃), 2.00 (3H, s, CH₃) and 1.98 (3H, s, CH₃); 13C NMR \( \delta_{\text{C}} \) (100 MHz; CDCl₃) 169.7, 169.5, 169.4 (C=O), 143.7 (C), 128.7, 127.9, 127.1, 98.4 (CH), 86.8 (C), 80.9, 74.4, 70.9 (CH), 63.2 (CH₂), 21.1, 20.6 and 20.5 (CH₃). 2 C and 14 CH equivalent.

1,2,3-Tri-O-acetyl-β-D-ribose

A solution of 1,2,3-tri-O-acetyl-5-trityl-β-D-ribose 48 (1.9 g, 3.67 mmol) in CH₂Cl₂ (48 mL), H₂O (1 mL) and TFA (1 mL) was stirred at room temperature for three hours. The reaction was extracted with H₂O (3 x 15 mL), dried (MgSO₄) filtered and evaporated to dryness. The residue was subject to flash chromatography on silica gel eluting with a gradient of hexane to hexane-EtOAc (7:3) then EtOAc-CH₃OH (9:1) to afford the alcohol 49 as a white foam (768 mg, 76%).
$^1$H NMR $\delta_{H}$ (270 MHz; CDCl$_3$) 6.00 (1H, d, $J$ 0.9, H-1), 5.26 (1H, dd, $J$ 6.9, 4.9, H-3), 5.21 (1H, dd, $J$ 4.9, 0.9, H-2), 4.12 (1H, ddd, $J$ 6.9, 4.0, 3.4, H-4), 3.69 (1H, dd, $J$ 12.4, 3.4, H-5a), 3.51 (1H, dd, $J$ 12.4, 4.0, H-5b), 2.00 (3H, s, CH$_3$), 1.97 (3H, s, CH$_3$) and 1.94 (3H, s, CH$_3$); $^{13}$C NMR $\delta_{C}$ (100 MHz; CDCl$_3$) 169.0, 168.5, 168.2 (C=O), 97.2, 81.3, 73.5, 68.8 (CH), 60.8 (CH$_2$), 20.1 (CH$_3$) and 19.5 (2 x CH$_3$).

$N,N$-diisopropylaminodichlorophosphine$^{48}$

$N,N$-diisopropylamine (141.0 mL, 1.0 mol) in dry ether (150 mL) was added dropwise to a stirred solution of phosphorus trichloride (44.0 mL, 0.5 mol) in dry ether (100 mL) at -78°C. The solution was diluted with dry ether (100 mL), vigorously stirred overnight at room temperature and the precipitated amine salt filtered, washed with ether (500 mL) and discarded. The organic phase was evaporated to dryness to afford a crude oil that was distilled (50-52°C; 0.5 mmHg, lit.$^{48}$ 62-63 °C; 7 mmHg) to afford the phosphine 68 as a clear colourless oil (72.0 g, 71 %).

$^1$H NMR $\delta_{H}$ (400 MHz; CDCl$_3$) 3.93 (2H, m, 2 x CH) and 1.20 (12H, m, 2 x CH) and 1.20 (12H, d, $J$ 7.0, 4 x CH$_3$); $^{31}$P NMR $\delta_{P}$ (162 MHz; CDCl$_3$) 169.5.

Bis(benzyloxy)diisopropylaminophosphine$^{53}$

Benzyl alcohol (3.1 mL, 29.4 mmol) in dry CH$_2$Cl$_2$ (25 mL) was added dropwise to a stirred solution of $N,N$-diisopropylaminodichlorophosphine 68 (3.0 g, 14.7 mmol) in dry CH$_2$Cl$_2$ (25 mL) and dry triethylamine (4.4 mL, 31.6 mmol) at -78°C. This was stirred for two hours at room temperature, then poured into aq. saturated NaHCO$_3$ (100 mL) and extracted with three portions of CH$_2$Cl$_2$ (3 x 50 mL). The combined organic layers were dried (Na$_2$SO$_4$), filtered, evaporated to dryness. The crude mixture was subject to flash chromatography on silica gel conditioned using
ether:hexane:Et$_3$N (74:25:1) (400 mL). The column was eluted using ether:hexane:Et$_3$N 74:25:1 to obtain the phosphine 52 as a clear oil (3.9 g, 79%).

R$_f$ 0.45 (ether:hexane 3:1); $^1$H NMR $\delta_H$ (400 MHz; CDCl$_3$) 7.42-7.30 (10H, m, H-Ar), 4.86-4.74 (4H, m, 2 x CH$_2$Ar), 3.82-3.71 (2H, m, 2 x CH), 1.29-1.25 (12H, dd, $J$ 6.7, 2.2, 4 x CH$_3$); $^{31}$P NMR $\delta_p$ (162 MHz; CDCl$_3$) 148.5.

Imidazolium triflate$^{50}$

![Imidazolium triflate](image)

To a stirred solution of imidazole (0.77g, 11.3 mmol) in CH$_2$Cl$_2$ (15 mL) at 0°C was added triflic acid (1.0 mL, 11.3 mmol). The reaction was warmed to room temperature and stirred for twenty minutes after which time ether (20 mL) was added. The white precipitate 51 was filtered dried under vacuum (2.2 g, 90%) and stored under nitrogen and used within twenty-four hours.

mp 197-198°C (from ether) (lit.$^{50}$ 197-198°C)

5-(Dibenzylphosphoryl)-1,2,3-tri-O-acetyl-$\beta$-D-ribose

![5-(Dibenzylphosphoryl)-1,2,3-tri-O-acetyl-$\beta$-D-ribose](image)

$^{51}$P NMR (162 MHz; CDCl$_3$) 148.5.

Bis(benzyloxy)diisopropylaminophosphine 52 (339 mg, 1.00 mmol) and imidazolium triflate 51 (436 mg, 2.00 mmol) were added to a solution of 1,2,3-tri-O-acetyl-$\beta$-D-ribose 49 (130 mg, 0.47 mmol) in CH$_2$Cl$_2$ (10 mL) and stirred for three hours. TLC analysis showed quantitative conversion to the phosphite intermediate (hexane:EtOAc 7:3 R$_f$ 0.3). The reaction was cooled to $-78$°C, $m$CPBA (517 mg, 3.00 mmol) was added and the oxidation was monitored by $^{31}$P NMR (139 ppm to $-0.69$ ppm) and TLC (hexane:EtOAc 1:1 R$_f$ 0.2). The reaction was quenched with 10% Na$_2$S$_2$O$_3$ (50 mL) and warmed to room temperature. The mixture was washed three times with aq. saturated NaHCO$_3$ (3 x 20 mL), brine (3 x 20 mL), dried (MgSO$_4$), filtered and evaporated to dryness. The product was then subject to flash chromatography on
silica gel using a gradient of hexane to hexane:EtOAc (1:1) as eluent to produce the protected phosphate 53 as a glassy solid (164 mg, 85%).

\[ ^1H \text{ NMR } \delta_H (400 \text{ MHz}; \text{CDCl}_3) 7.36-7.34 (10H, m, H-Ar), 6.15 (1H, s, H-1), 5.39 (1H, dd, \text{J} 7.2, 4.9, H-3), 5.34 (1H, d, \text{J} 4.9, H-2), 5.06 (2H, t, \text{J} \text{PH} 2.5, \text{CH}_2\text{Ar}), 5.04 (2H, t, \text{J} \text{PH} 2.5, \text{CH}_2\text{Ar}), 4.33 (1H, m, H-4), 4.18 (1H, ddd, \text{J} \text{HH} 11.3, 6.2, \text{J} \text{PH} 2.5, H-5_a), 4.09 (1H, ddd, \text{J} \text{HH} 11.3, 6.3, \text{J} \text{PH} 2.5, H-5_b), 2.12 (3H, s, \text{CH}_3), 2.04 (3H, s, \text{CH}_3) \text{ and } 1.99 (3H, s, \text{CH}_3); ^{13}\text{C NMR } \delta_C (100 \text{ MHz}; \text{CDCl}_3) 169.6, 169.4, 169.2 (\text{C=O}), 135.7, 135.6 (\text{C}), 128.6, 128.0 (10 x \text{CH}), 98.0, 79.8, 74.1, 70.1 (\text{CH}), 69.5, 69.4, 66.3 (\text{CH}_2), 20.6, 20.5 \text{ and } 20.4 (\text{CH}_3); ^{31}\text{P NMR } \delta_P (109 \text{ MHz}; \text{CDCl}_3) -0.52.\]

1,2,3-Tri-O-acetyl-β-D-ribose-5-phosphate

To a stirred solution of 5-bis(benzylphosphate)-1,2,3-tri-O-acetylribose 53 (75 mg, 0.15 mmol) in CH\(_3\)OH (5 mL) and H\(_2\)O (0.25 mL) was added 20% Pd(OH)\(_2\)/C (50 mg) and cyclohexene (2 mL, 20 mmol). This was heated to 80°C for three hours after which time the catalyst was removed using a membrane filter and the product evaporated to dryness under vacuum to afford the free phosphate 50 as a white foam (27 mg, 50%).

\[ ^{31}\text{P NMR } \delta_P (109 \text{ MHz}; \text{CD}_3\text{OH}) 0.69; m/z [\text{FAB}] 355.0 (M^+, 100%). \]

\(^1\text{H}\) and \(^{13}\text{C}\) NMR proved inconclusive.
7.3 Synthesis of protected adenosine diphosphate

*Bis(2-cyanoethoxy)diisopropylaminophosphine*[^49]

\[
\begin{array}{c}
\text{N} \quad \text{P} \\
\text{O} \quad \text{CN} \\
\text{O} \quad \text{CN}
\end{array}
\]

[^69]

Hydroxypropionitrile (4.4 mL, 64.5 mmol) in dry CH\(_2\)Cl\(_2\) (50 mL) was added dropwise to a stirred solution of *N,N*-diisopropylaminodichlorophosphine 68 (6.5 g, 32.3 mmol) in dry CH\(_2\)Cl\(_2\) (50 mL) and dry triethylamine (9.6 mL, 69.4 mmol) at \(-78^\circ\text{C}\). This was stirred for two hours at room temperature, poured into aq. saturated NaHCO\(_3\) (200 mL) and extracted with three times with CH\(_2\)Cl\(_2\) (3 x 100 mL). The combined organic extracts were dried (Na\(_2\)SO\(_4\)), filtered, evaporated to dryness. The resulting oil was subject to flash chromatography on silica gel conditioned using ether:hexane:Et\(_3\)N (74:25:1) (400 mL) then ether:hexane 3:1 (400 mL) before loading. The column was eluted using ether:hexane 3:1 to obtain the phosphine 69 as a clear oil (6.5 g, 74%).

R\(_f\) 0.61 (ether:hexane 3:1); \(^1\text{H} \text{NMR} \delta_{\text{H}} \text{(400 MHz; CDCl} \text{)}_3 \text{) 3.92–3.77 (4H, m, 2 x CH}_2\text{CN), 3.68–3.44 (2H, m, 2 x CH), 2.65 (4H, t, J 6.2, 2 x CH}_2\text{O), 1.18 (12H, d, J 7.0, 4 x CH}_3\text{); }^{31}\text{P NMR} \delta_{\text{P}} \text{(162 MHz; CDCl} \text{)}_3 \text{) 149.6.}

3',5'-O-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)adenosine[^45,^46]

\[
\begin{array}{c}
\text{S} \quad \text{O} \\
\text{O} \quad \text{N} \quad \text{H}_2
\end{array}
\]

1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (1.20 mL, 3.75 mmol) was added to a solution of adenosine 58 (1.0 g, 3.75 mmol) in pyridine (40 mL) and stirred for three hours at room temperature. The solvent was removed under vacuum and the product dissolved in CH\(_2\)Cl\(_2\) (20 mL) washed with aq. saturated NaHCO\(_3\) (3 x 20 mL), dried (Na\(_2\)SO\(_4\)), filtered and evaporated to dryness. The compound was then subject to
flash chromatography on silica gel using CH$_2$Cl$_2$-CH$_3$OH as eluent to afford the cyclic protected adenosine 65 as a white solid (1.5 g, 80%).

R$_f$ 0.34 (CH$_2$Cl$_2$;CH$_3$OH 95:5); mp 95-96 °C (from CH$_2$Cl$_2$) (lit. 46 97-99 °C); [α]$^2_D$ = 33 (c 1 in CHCl$_3$); $^1$H NMR δ$_H$ (400 MHz; CDCl$_3$) 8.29 (1H, s, H-2a), 8.03 (1H, s, H-8a), 6.01 (1H, d, J 0.8, H-1'), 4.99 (1H, dd, J 8.2, 5.4, H-3'), 4.56 (1H, dd, J 5.4, 0.8, H-2'), 4.19-4.13 (2H, m, H-4, H-5'a), 4.04 (1H, m, H-5'b) and 1.12-1.04 (28H, m, 4 x CH(CH$_3$)$_2$); $^{13}$C NMR δ$_C$ (100 MHz; CDCl$_3$) 155.6 (C), 152.8 (CH), 149.1 (C), 139.5 (CH), 120.4 (C), 90.1, 82.3, 75.4, 70.7 (CH), 61.8 (CH$_2$), 17.9, 17.82, 17.80, 17.76, 17.54, 17.46, 17.43, 17.36 (CH$_3$), 13.8, 13.5, 13.2 and 13.0 (CH); m/z [FAB$^+$] 510.2, (M+H)$^+$, 74% [found 510.2571 C$_{22}$H$_{46}$N$_5$O$_5$Si$_2$ requires 510.2568].

2'-(Di(2-cyanoethoxyphosphoryl))-3',5'-O-(1,1,3,3-tetraisopropylsiloxane-1,3-diyl) adenosine

2'-(Di(2-cyanoethoxyphosphoryl))-3',5'-O-(1,1,3,3-tetraisopropylsiloxane-1,3-diyl) adenosine

$^{Bis}$(2-cyanoethoxy)diisopropylaminophospine 69 (455 mg, 1.68 mmol) and imidazolium triflate 51 (976 mg, 4.48 mmol) were added to a solution of 3', 5' protected adenosine 65 (522 mg, 1.03 mmol) in CH$_2$Cl$_2$ (30 mL) and stirred under a nitrogen atmosphere. After three hours TLC analysis showed quantitative conversion to the phosphite intermediate (CHCl$_3$;acetone 2:8; R$_f$ 0.45 P$^{III}$). The reaction was cooled to −78°C, mCPBA (293 mg, 1.7 mmol) added and the oxidation was monitored by $^{31}$P NMR (139.6 ppm to −1.3 ppm), then quenched with 10% Na$_2$S$_2$O$_3$ (50 mL) and warmed to room temperature. The mixture was washed with aq. saturated NaHCO$_3$ (3 x 20 mL) and brine (3 x 20 mL), dried (Na$_2$SO$_4$), filtered and evaporated to dryness. The product was then subject to flash chromatography on silica gel using chloroform-acetone as eluent to afford the protected phosphate adenosine 71 as a white solid (699 mg, 98%).
Rf 0.34 P\(^{(V)}\) (CHCl\(_3\):acetone 2:8); mp 168-170 °C (from CHCl\(_3\)); \([\alpha\]\(^{25}_D\) = -24 (c 1 in CHCl\(_3\)); \(^1\)H NMR \(\delta_H\) (400 MHz; CDCl\(_3\)) 8.24 (1H, s, H-2\(\alpha\)), 8.12 (1H, s, H-8\(\alpha\)), 6.18 (1H, s, H-1'), 5.30 (1H, m, H-2'), 4.92 (1H, m, H-3'), 4.50-4.35 (4H, m, 2 x CH\(_2\)CH\(_2\)O), 4.26 (1H, dd, J 13.4, 2.4, H-5\(\alpha\)), 4.15 (1H, m, H-4'), 4.03 (1H, dd, J 13.4, 2.4, H-5\(\beta\)), 2.90 (2H, t, J 6.2, CH\(_2\)CN), 2.81 (2H, t, J 6.2, CH\(_2\)CN) and 1.20-1.02 (28H, m, 4 x CH(CH\(_3\))\(_2\)); \(^{13}\)C NMR \(\delta_C\) (100 MHz; CDCl\(_3\)) 155.3 (C), 152.8 (CH), 148.9 (C), 138.8 (CH), 120.2, 116.3, 116.0 (C), 88.2, 81.2, 80.7, 68.0 (CH), 62.7, 62.6, 59.4, 19.7, 19.6 (CH\(_2\)), 17.4, 17.2 (3 x CH\(_3\)), 16.97, 16.88, 16.85, 16.81 (CH\(_3\)), 13.4, 12.9, 12.8, 12.5 (CH); \(^{31}\)P NMR \(\delta_P\) (162 MHz; CDCl\(_3\)) -1.30; \(\text{m/z}\) [FAB\(^{+}\)] 696.2 (M+H\(^+\)), 100% [found 696.2760 C\(_{28}\)H\(_{47}\)N\(_7\)O\(_8\)PSi\(_2\) requires 696.2762].

2'-\((\text{Di}(2\text{-cyanoethoxyphosphoryl}))-3'-\text{O-}(3\text{-hydroxy-1,1,3,3-tetraisopropylsiloxane-1-yl})\) adenosine

\(72\)

A solution of 2' phosphate adenosine \(71\) (689 mg, 0.99 mmol) in TFA-H\(_2\)O-THF (1:1:4; \(v/v/v\)) (60 mL) was stirred at 0°C for three hours. The reaction was neutralised with aq. saturated NaHCO\(_3\) (60 mL) and the organic phase washed with H\(_2\)O (3 x 20 mL) and brine (3 x 20 mL), dried (Na\(_2\)SO\(_4\)), filtered and evaporated to dryness to yield the selectively deprotected hydroxyl \(72\) as a white solid (705 mg, 100%).

Rf 0.19 (CHCl\(_3\):acetone 2:8); mp 142-144 °C (from THF); \([\alpha\]\(^{25}_D\) = -52 (c 1 in CHCl\(_3\)); \(^1\)H NMR \(\delta_H\) (400 MHz; CDCl\(_3\)) 8.43 (1H, s, H-2\(\alpha\)), 8.22 (1H, s, H-8\(\alpha\)), 6.31 (1H, d, J 6.2, H-1'), 5.59 (1H, m, H-2'), 4.89 (1H, m, H-3'), 4.34 (1H, d, J 2.0, H-4'), 4.26-4.06 (4H, m, 2 x CH\(_2\)O), 3.92 (1H, dd, J 12.5, 2.0, H-5\(\alpha\)), 3.40 (1H, dd, J 12.5, 2.0, H-5\(\beta\)), 2.78 (2H, dd, J 10.1, 5.4, CH\(_2\)CN), 2.73 (2H, dd, J 10.1, 5.4, CH\(_2\)CN) and 1.15-1.01 (28H, m, 4 x CH(CH\(_3\))\(_2\)); \(^{13}\)C NMR \(\delta_C\) (100 MHz; CDCl\(_3\)) 160.2 (C), 156.5 (CH), 152.8 (C), 144.7 (CH), 123.7 (C), 121.0 (2 x C), 91.5, 91.3, 82.7, 75.6 (CH), 67.6, 67.6, 65.4, 23.1, 23.0 (CH\(_2\)), 21.0 (3 x CH\(_3\)), 20.93 (2 x CH\(_3\)), 20.91, 20.84, 20.80.
(CH₃), 17.8 (2 x CH), 17.52, 17.50 (CH); ³¹P NMR δₚ (162 MHz; CDCl₃) -1.80; m/z [FAB⁺] 714.4 (M+H)⁺, 82% [found 714.2856 C₂₈H₄₉N₇O₉PSi₂ requires 714.2868].

5′-(Dibenzylphosphoryl)-2′-(di(2-cyanoethoxyphosphoryl))-3′-O-(3-hydroxy-1,1,3,3-tetraisopropylsiloxane-1-yl) adenosine

Bis(benzyl)diisopropylaminophosphine 52 (1.07 g, 3.15 mmol) and imidazolium triflate 51 (1.83 g, 8.40 mmol) were added to a solution of 5′ alcohol 72 (1.50 g, 2.10 mmol) in CH₂Cl₂ (50 mL) and stirred overnight after which time TLC showed complete conversion to phosphite intermediate (CHCl₃:acetone 2:8; Rf 0.3 P(II)). The reaction was cooled to -78°C, mCPBA (1.35 g, 6.0 mmol) added and the oxidation monitored by ³¹P NMR (140.4 ppm to -2.0 ppm), then quenched with 10% Na₂S₂O₃ (100 mL) and warmed to room temperature. The mixture was washed with aq. saturated NaHCO₃ (3 x 20 mL) and brine (3 x 20 mL), dried (Na₂SO₄), filtered and evaporated to dryness. The product was then subject to flash chromatography on silica gel using chloroform-acetone as eluent to afford the diphosphate 73 as a white solid (1.55 g, 76%).

Rf 0.23 P(V) (CHCl₃:acetone 2:8); mp 157-159 °C (from CHCl₃); ¹H NMR δH (400 MHz; CDCl₃) 8.16 (1H, s, H-2), 8.08 (1H, s, H-8), 7.28-7.14 (10H, m, H-Ar), 6.19 (1H, d, J 3.7, H-1′), 5.41(1H, m, H-2′), 4.96-4.88 (5H, m, 2 x (OCH₂Ph), H-3′), 4.38-3.97 (7H, m, 2 x (CH₂CH₂O), H-4′, 5′ₐ, H-5′ₖ), 2.64-2.55 (4H, m, 2 x CH₂CN) and 1.01-0.72 (28H, m, 4 x CH(CH₃)₂); ¹³C NMR δC (100 MHz; CDCl₃) 155.9 (C), 153.1 (CH), 149.4 (C), 139.4 (CH), 135.6 (C), 135.5 (C), 128.88, 128.87, 128.81, 128.79, 128.20, 128.17 (CH), 120.0, 116.7, 116.4 (C), 87.0, 82.9, 79.1 (CH), 70.1, 70.0 (CH₂), 70.0 (CH), 68.2, 66.4, 66.3, 20.0, 19.9 (CH₂), 17.81, 17.75, 17.70, 17.68, 17.61 (CH₃), 14.0, 13.9, 13.8 and 13.7 (CH), 4 aromatic CH and 3 silanol CH₃ occluded; ³¹P NMR
δP (162 MHz; CDCl₃) -2.00 and -0.42; m/z [FAB⁺] 974.9 (M⁺,H⁺, 56%) [found 974.3447 C₄₂H₆₂N₇O₁₂P₂Si₂ requires 974.3470].

2’-(Di(2-cyanoethoxyphosphoryl))-3’-O-(3-hydroxy-1,1,3,3-tetraisopropylsiloxane-1-yl) adenosine-5’-phosphate

To a stirred mixture of 2’, 5’ phosphorylated adenosine 73 (300 mg, 0.304 mmol) in CH₃OH (10 mL) and water (0.5 mL) was added 20% Pd(OH)₂/C (100 mg) and cyclohexene (3.5 mL). The reaction was heated to 80°C for three hours and monitored by ³¹P NMR (-0.42 ppm to 1.03 ppm) for completion. After cooling the reaction mixture was passed through a membrane filter and the catalyst washed copiously with a methanol / water mixture (1:1). Concentration of the filtrate afforded the deprotected phosphate 74 as a white solid (200 mg, 82%).

mp 139–141 °C (from methanol); [α]²⁵D -14° (c 0.36 in Methanol); ¹H NMR δH (270 MHz; CD₃OD) 8.65 (1H, s, H-2αd), 8.25 (1H, s, H-8αd), 6.32 (1H, d, J 5.9, H-1’), 5.33 (1H, m, H-2’), 4.82 (1H, m, H-3’), 4.41 (1H, m, H-4’), 4.16-4.07 (6H, m, 2 x CH₂O, H-5’α, H-5’β), 2.76-2.67 (4H, m, 2 x CH₂CN) and 1.05-0.75 (28H, m, 4 x CH(CH₃)₂);

¹³C NMR δC (100 MHz; CDCl₃) 150.8, 147.7 (C), 146.4 (CH), 141.2 (CH), 118.8 117.4, 117.1 (C), 88.1, 81.9, 80.5, 71.5 (CH), 67.3, 63.9, 62.1, 19.1, 19.0 (CH₂), 16.9, 16.84, 16.75 (CH₃), 16.62 (2 x CH₃), 16.55, 16.51, 16.3 (CH₃), 13.9, 13.7, 13.4, 13.2 (CH); ³¹P NMR δP (109 MHz; CDCl₃) -2.40 and 1.03; m/z [FAB⁺] 794.3 (M+H)⁺, 100% [found 794.2548 C₂₈H₅₀N₇O₁₂P₂Si₂ requires 794.2531].
7.4 Synthesis of the pyrophosphate bond
Adenosine-5’-monophosphate morpholminate \(^{61}\)

![Chemical structure of morpholinate 75](image)

Powdered triphenylphosphine (131 mg, 0.5 mmol), 2,2'-dipyridyl disulphide (110 mg, 0.5 mmol) and morpholine (87 µL, 1.0 mmol) were added to a stirred solution of adenosine 5'-monophosphate 58 (free acid) (69 mg, 0.2 mmol) in DMSO (1.0 mL). The reaction was stirred at room temperature and monitored using ion exchange HPLC. After three hours another portion triphenylphosphine (68 mg, 0.25 mmol) and 2,2'-dipyridyl disulphide (55 mg, 0.25 mmol) were added and stirred for a further hour. The reaction was quenched using 0.1 M NaI in acetone (10 mL) and the white precipitate filtered, washed with acetone until any yellow discoloration had been removed. The product was dried under vacuum over phosphorous pentoxide to afford the morpholinate 75 as a white solid (83 mg, 100 %).

\(^1\)H NMR \(\delta_H\) (400 MHz; D\(_2\)O) 8.21 (1H, s, H-2\(a\)d), 8.00 (1H, s, H-8\(a\)d), 5.88 (1H, d, \(J = 5.1\), H-1'), 4.58 (1H, m, H-2'), 4.30 (1H, t, \(J = 4.6\)), H-3'), 4.13 (1H, br s, H-4'), 3.89-3.82 (2H, m, H-5\(a\)'s, H-5\(b\)'s), 3.32 (4H, t, \(J = 4.7\), 2 x CH\(_2\)N) and 2.69 (4H, t, \(J = 4.7\), 2 x CH\(_2\)CH\(_2\)O); \(^{31}\)P NMR \(\delta_P\) (162 MHz; D\(_2\)O) 8.77. Identical to commercially available compound.
Nicotinamide adenine dinucleotide

β-NMN 21 (25 mg, 0.075 mmol) and MgSO₄·7H₂O (34 mg, 0.136 mmol) were added to a solution of AMP morpholinate 75 (28 mg, 0.068 mmol) in 0.2 M MnCl₂* in formamide (563 μL, 0.1125 mmol) under a nitrogen atmosphere. The solution was stirred at room temperature for eighteen hours after which time RP ion pair HPLC analysis showed completion of the reaction. The mixture was applied to a column of Q-Sepharose and eluted with a gradient of water–1 M TEAB (eluting at 5-8%). The required fractions were analysed by reverse phase HPLC, collected, treated with chelex resin, filtered, concentrated under vacuum and lyophilised from D₂O to afford NAD 6 as a white hygroscopic powder (12 mg, 27 %).

* MnCl₂ dissolved in formamide and left over 4 Å molecular sieves for three days. Rₜ 7.5 minutes (RP-HPLC 94 % aq. 0.1 M KH₂PO₄, 6 mM tetrabutylammonium bromide and 6% CH₃OH; Isocratic for 30 mins); ¹H NMR δH (270 MHz; D₂O) 9.29 (1H, s, H-2'ₐd), 9.11 (1H, d, J 6.1, H-6ₐc), 8.78 (1H, d, J 8.2, H-4ₐc), 8.37, (1H, s, H-2'ₐd), 8.15 (1H, m, H-5ₐc), 8.05 (1H, s, H-8'ₐd), 6.04 (1H, d, J 5.2, H-1'), 5.98 (1H, d, J 5.8, H-1), 4.72 (1H, t, J 5.8, H-2) and 4.51-4.21 (9H, m, H-3, H-4, H-5, H-2', H-3', H-4', H-5'); ¹³C NMR δC (100MHz; D₂O) 165.1, 155.1 (C), 152.5 (CH), 148.7 (C), 145.7, 142.3, 139.8, 139.6 (CH), 133.5 (C), 128.5 (CH), 118.2 (C), 99.9, 86.9, 86.5, 83.7, 77.5, 73.9, 70.6, 70.3 (CH), 65.3 and 64.8 (CH₂); ³¹P NMR δP (162 MHz; D₂O) -11.5; m/z [FAB'] 663.3 (M⁺, 35%). Identical to commercial sample.
Nicotinamide adenine dinucleotide 2'(2-cyanoethoxy) phosphate

Carbonyl diimidazole (48 mg, 0.3 mmol) and Et₃N (14 μL, 0.1 mmol) were added to a solution of β-NMN 21 (33 mg, 0.1 mmol) in DMF (300 μL). This was stirred at room temperature under a nitrogen atmosphere for three hours and monitored by ³¹P NMR (−9.5 ppm). Once complete a few drops of methanol were added and the solution stirred for a further five minutes. The solution was evaporated to dryness, co-evaporated with DMF (3 x 100 μL) to afford a yellow oil, to which was added a solution of 5' phosphate adenosine 74 (63 mg, 0.08 mmol) in DMF (300 μL) and the resulting mixture stirred at room temperature overnight. The reaction was then quenched with 25 mM NH₄OAC (6 mL) and extracted with CH₂Cl₂ (3 x 5 mL). The aqueous phase was evaporated to dryness and re-suspended in a solution of 10 % aq. TFA (10 mL) and EtOAc (0.5 mL). This was stirred for three hours and monitored by reverse phase TLC for the disappearance of starting material. The reaction was evaporated to dryness, re-suspended in H₂O, loaded onto an ion exchange gradifac column (AG-MP1) and eluted using a linear gradient of 150 mM aq. TFA. Fractions analysed by ion exchange HPLC (method A) and the appropriate fractions were concentrated and lyophilised from D₂O to afford the pyrophosphate 86 as a white hygroscopic powder (6 mg, 10 %).

R₁ 11 minutes (AG-MP1 water:150 mM aq. TFA); ¹H NMR δH (600 MHz; D₂O) 9.27 (1H, s, H-2ₐnic), 9.11 (1H, d, J 6.3, H-6ₐnic), 8.79 (1H, d, J 8.0, H-4ₐnic), 8.44 (1H, s, H-2′ₐad), 8.29 (1H, s, H-8′ₐad), 8.14 (1H, m, H-5ₐnic), 6.15 (1H, s, H-1′), 6.01 (1H, s, H-1), 5.00 (1H, m, H-2′), 4.53 (1H, m, H-3′), 4.44-4.41 (2H, m, H-2, H-3), 4.34 (1H, m, H-4), 4.27-4.10 (5H, m, H-4′, 5′, 5), 3.81 (2H, m, CH₂CH₂O), 2.58 (2H, m, CH₂CN); ¹³C...
Nicotinamide adenine 3'-O-(3-hydroxy-1,1,3,3-tetraisopropylsiloxane-1-yl) dinucleotide 2'-(2-cyanoethoxy) phosphate

Carbonyl diimidazole (107 mg, 0.60 mmol) and Et₃N (34 µL, 0.24 mmol) were added to a solution of β-NMN 21 (75 mg, 0.22 mmol) in DMF (750 µL). This was stirred at room temperature under a nitrogen atmosphere for three hours and monitored by ³¹P NMR (−9.5 ppm). Once complete a few drops of methanol were added and the solution stirred for a further five minutes. The solution was then evaporated to dryness and co-evaporated with DMF (3 x 100 µL). A solution of 5' phosphate adenosine 74 (140 mg, 0.17 mmol) in DMF (700 µL) was added to the residue and the reaction stirred at room temperature overnight. The reaction was evaporated to dryness under reduced pressure, suspended in H₂O (5 mL), extracted with CH₂Cl₂ (3 x 5 mL) and loaded onto Q sepharose fast flow resin. The compound was eluted using a linear gradient of 1 M aq. TEAB. Fractions were analysed by ion exchange HPLC (method A) and the appropriate fractions evaporated to dryness. The white powder was then re-suspended in CH₃OH, repeatedly co-evaporated with CH₃OH to
remove excess Et$_3$N and lyophilised from D$_2$O to afford the pyrophosphate triethylammonium salt 85 as a white hygroscopic powder (129 mg, 63 %). 

R$_t$ 11 minutes (AG-MP1 water:150 mM aq. TFA); $^1$H NMR $\delta$H (400 MHz; CD$_3$OD) 9.32 (1H, s, H-2$_{nic}$), 9.12 (1H, d, J 6.6, H-6$_{nic}$), 8.63 (1H, d, J 6.6, H-4$_{nic}$), 8.27 (1H, s, H-2'$_{ad}$), 7.94 (1H, t, J 6.6, H-5$_{nic}$), 7.86 (1H, s, H-8'$_{ad}$), 6.00 (1H, d, J 6.2, H-1'), 5.81 (1H, d, J 5.1, H-1), 4.98 (1H, m, H-2'), 4.24 (1H, t, J 5.1, H-2), 4.17-3.97 (7H, m, H-3, H-4, H-5, H-4', H-5'), 3.55 (2H, m, CH$_2$CH$_2$O), 2.22 (2H, dd, $J_{HH}$ 10.9, $J_{PH}$ 6.2, CH$_2$CN) 1.33-0.95 (28H, m, (CH(CH$_3$)$_3$)$_4$). H-3' occluded by HDO peak at 4.60; $^{13}$C NMR $\delta$C (100MHz; CD$_3$OD) 163.9, 155.5, 152.2 (C), 149.8, 145.6, 142.9, 140.9, 140.5 (CH), 134.3 (C), 128.3 (CH), 118.8, 117.8 (C), 100.7, 88.3, 85.8, 85.6, 78.2, 77.4, 72.9, 71.3 (CH), 66.0, 65.0, 60.5, 19.0 (CH$_2$), 17.14 (4 x CH$_3$), 17.09 (2 x CH$_3$), 17.0 (2 x CH$_3$), 14.0, 13.9, 13.8 and 13.1 (CH); $^{31}$P NMR $\delta$P (109 MHz; D$_2$O) -0.82 and -10.95; m/z [FAB$^+$] 1055.2 (M$^+$, 40 %).

**Cyclic-2',3'-phosphoryl-adenosine-5'-phosphate**

![Chemical Structure](image)

To a solution of 5' phosphate adenosine 74 (50 mg, 0.063 mmol) in THF (1 mL) at 0°C buffered with AcOH (6 $\mu$L) was added 1 N TBAF in THF (120 $\mu$L). The solution was stirred under a nitrogen atmosphere for three hours, evaporated to dryness, re-suspended in water (10 mL) and extracted with CH$_2$Cl$_2$ (3 x 10 mL). The aqueous phase was applied to a Q sepharose fast flow column and eluted with a gradient of water to 1 M aq. TEAB and analysed by ion exchange HPLC. Fractions eluting at 80% TEAB (R$_t$ 14.9 min) were collected and evaporated to dryness under reduced pressure. The white powder was then re-suspended in CH$_3$OH, repeatedly co-evaporated with CH$_3$OH to remove excess Et$_3$N and lyophilised from D$_2$O to afford the cyclic phosphate 87 as a white hydroscopic powder (8 mg, 32%).
Rt 15 min (AG-MP1 water:150 mM aq. TFA); ^1H NMR δ_H (400 MHz; D_2O) 8.23 (1H, s, H-2), 8.08 (1H, s, H-8), 6.17 (1H, d, J 3.9, H-1'), 5.28 (1H, m, H-2'), 5.04 (1H, m, H-3'), 4.45 (1H, m, H-4') and 4.00-3.97 (2H, m, H-5'); ^31P NMR δ_P (109 MHz; D_2O) 20.7 and 1.2; m/z [FAB'] 408.1 (M', 70%).

Nicotinamide adenine dinucleotide 2'-(2-cyanoethoxy) phosphate

To a solution of purified pyrophosphate (TEA salt) 24 (73 mg, 0.063 mmol) in THF (700 µL) at 0°C were added AcOH (4 µL) and 1N TBAF in THF (80 µL). The solution was stirred for three hours, then evaporated to dryness under reduced pressure, re-suspended in water (10 mL) and extracted with CH_2Cl_2 (3 x 10 mL). The aqueous phase was loaded onto a column of AG-MP1 resin, eluted using a gradient of water:150 mM aq. TFA and analysed by ion exchange HPLC. The desired fractions were then evaporated to dryness and lyophilised from D_2O to afford desilylated pyrophosphate 23 as a white hydroscopic powder (37 mg, 75%).

Rt 11 minutes (AG-MP1 water:150 mM aq. TFA); ^1H NMR δ_H (400 MHz; D_2O) 9.24 (1H, s, H-2_nic), 9.08 (1H, d, J 6.6, H-6_nic), 8.76 (1H, d, J 7.8, H-4_nic), 8.41 (1H, s, H-2_ad), 8.27 (1H, s, H-8_ad), 8.10 (1H, dd, J 7.8, 6.6, H-5_nic), 6.13 (1H, d, J 5.0, H-1'), 5.98 (1H, d, J 5.4, H-1), 4.96 (1H, m, H-2'), 4.50 (1H, m, H-3'), 4.41-4.38 (2H, m, H-2, 3), 4.30 (1H, m, H-4'), 4.24-4.04 (5H, m, H-4, H-5', H-5), 3.81-3.73 (2H, m, CH_2CH_2O), 2.56-2.53 (2H, m, CH_3CN); ^31P NMR δ_P (109 MHz; D_2O) -0.25 and -10.70. m/z [FAB'] 795.1 (M', 60%) [found 795.0953 C_{24}H_{30}N_8O_{17}P_3 requires 795.0942].
Adenosine-2',5'-bisphosphate

Method One

$N,O$-Bis(trimethylsilyl)acetamide (232 µL, 0.94 mmol) and DBU (23 µL, 0.15 mmol) were added to a solution of 5' phosphatase adenosine 74 (20 mg, 0.025 mmol) in pyridine (2.5 mL). It was stirred under a nitrogen atmosphere for ninety minutes, after which time the solvent was removed under reduced pressure, re-suspended in water (5 mL) and extracted with ether (3 x 10 mL). The aqueous phase was evaporated to dryness, co-evaporated with toluene and suspended in THF (500 µL). 1 N TBAF in THF (50 µL) was added and the reaction stirred for two hours at 0°C under a nitrogen atmosphere. This was evaporated to dryness under reduced pressure, re-suspended in water (5 mL), extracted with CH$_2$Cl$_2$ (3 x 10 mL) and loaded onto a column of AG-MP1 resin. Fractions were analysed by ion exchange HPLC (method A) and the desired fractions were evaporated to dryness and lyophilised from D$_2$O to afford 2',5'-ADP 87 as a white hydroscopic powder (4 mg, 36%).

Method Two

$N,O$-Bis(trimethylsilyl)acetamide (185 µL, 0.75 mmol) and DBU (18 µL, 0.12 mmol) were added to a solution of purified pyrophosphate 85 (20 mg, 0.017 mmol) in pyridine (2.0 mL). It was stirred under a nitrogen atmosphere for ninety minutes, after which time the solvent was removed under reduced pressure, re-suspended in water (5 mL) and extracted with ether (3 x 10 mL). The aqueous phase was evaporated to dryness and co-evaporated with toluene and suspended in THF (500 µL). 1 N TBAF in THF (50 µL) was added and the reaction stirred for two hours at 0°C under a nitrogen atmosphere. This was evaporated to dryness under reduced pressure, re-suspended in water (5 mL), extracted with CH$_2$Cl$_2$ (3 x 10 mL) and loaded onto a column of AG-MP1 resin. Fractions were analysed by ion exchange
HPLC and the desired fractions were evaporated to dryness to afford 2',5'-ADP 87 as a clear glassy solid (3 mg, 42%).

R<sub>t</sub> 14 minutes (AG-MP1 water:150 mM aq. TFA); <sup>1</sup>H NMR δ<sub>H</sub> (400 MHz; D<sub>2</sub>O) 8.45 (1H, s, H-2), 8.26 (1H, s, H-8), 6.16 (1H, d, J 5.8, H-1'), 4.97 (1H, m, H-2'), 4.47 (1H, m, H-3'), 4.27 (1H, m, H-4') and 4.00-3.99 (2H, m, H-5'); <sup>31</sup>P NMR δ<sub>P</sub> (109 MHz; D<sub>2</sub>O) 1.1 and 0.6.

7.5 Modified synthesis of 2', 5' adenosine diphosphate

2'-(Dibenzyloxyphosphoryl)-3',5'-C>(3-hydroxy-1,1,3,3-tetraisopropyldisiloxane-1,3-diyl) adenosine

Bis(benzyloxy)diisopropylaminophosphine 52 (499 mg, 1.47 mmol) and imidazolium triflate 51 (856 mg, 3.98 mmol) were added to a solution of 3',5' protected adenosine 65 (500 mg, 0.98 mmol) in CH<sub>2</sub>C<sub>1</sub> (30 mL) under a nitrogen atmosphere and stirred overnight. TLC analysis showed quantitative conversion to the phosphite intermediate (CHCl<sub>3</sub>:acetone 1:1 R<sub>f</sub> 0.6). The reaction was cooled to -78°C, mCPBA (508 mg, 2.95 mmol) was added and the oxidation monitored by <sup>31</sup>P NMR (149 ppm to -0.77 ppm) and TLC (CHCl<sub>3</sub>:acetone 2:8 R<sub>f</sub> 0.6). The reaction was quenched with 10% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (50 mL) and warmed to room temperature. The mixture was washed with NaHCO<sub>3</sub> (3 x 20 mL), brine (3 x 20 mL), dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and evaporated to dryness. The product was then subject to flash chromatography on silica gel using CH<sub>2</sub>Cl<sub>2</sub> to CH<sub>2</sub>Cl<sub>2</sub>:CH<sub>3</sub>OH (95:5) as the eluent to afford the protected phosphate 93 as a white solid (591 mg, 78%).

R<sub>f</sub> 0.4 (CH<sub>2</sub>Cl<sub>2</sub>:CH<sub>3</sub>OH 95:5); mp 160-162 °C (from CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR δ<sub>H</sub> (600 MHz; CDCl<sub>3</sub>) 8.20 (1H, s, H-2a), 7.82 (1H, s, H-8a), 7.33-7.30 (10H, m, H-Ar), 6.00 (1H, s, H-1'), 5.73 (2H, s, NH<sub>2</sub>), 5.33 (1H, dd, J<sub>HH</sub> 8.2, J<sub>PH</sub> 4.9, H-2'), 5.17-5.07 (5H, m, 2 x OCH<sub>2</sub>Ph, H-3'), 4.16 (1H, dd, J 13.2, 2.0, H-5'<sub>a</sub>), 4.06 (1H, dt, J 2.6, 2.0, H-4'), 4.00 (1H, dd, J 13.2, 2.6, H-5'<sub>b</sub>) and 1.10-1.00 (28H, m, 4 x CH(CH<sub>3</sub>)<sub>2</sub>); <sup>13</sup>C NMR δ<sub>C</sub>
(150 MHz; CDCl₃) 155.6 (C), 153.2 (CH), 149.3 (C), 139.6 (CH), 135.9, 135.7 (C), 128.77, 128.76, 128.73, 128.71, 128.13, 128.08 (CH), 120.4 (C), 88.8, 81.5, 79.8 (CH), 69.9, 69.8 (CH₂), 68.8 (CH), 60.1 (CH₂), 17.6, 17.49, 17.48, 17.46, 17.2, 17.0 (CH₃), 13.5, 13.2, 13.0 and 12.8 (CH), 4 aromatic CH and 2 silanol CH₃ occluded; ³¹P NMR δₚ (162 MHz; CDCl₃) -0.77 m/z [FAB⁺] 770.3 (M⁺, 100%) [found 770.3156 C₃₆H₅₃N₅O₈PSi₂⁺ requires 770.3170].

2′-(Dibenzyloxyphosphoryl)-3′-O-(3-hydroxy-1,1,3,3-tetraisopropylidisiloxane-1-yl) adenosine

Benzyl protected phosphate adenosine 93 (591 mg, 0.77 mmol) at 0 °C was dissolved in TFA−H₂O−THF (1:1:4; v/v/v, 60 mL, 0 °C) and stirred for four hours at 0 °C after which TLC analysis (CH₂Cl₂:CH₃OH, 9:1; Rf 0.55) showed the reaction had gone to completion. It was neutralised with aq. saturated NaHCO₃ and the organic layer extracted with H₂O (3 x 20 mL) and brine (3 x 20 mL). The organic phase was collected, dried (Na₂SO₄), filtered and evaporated to dryness to afford the deprotected hydroxyl 94 as a white solid (456 mg, 76%).

mp >210°C (decomposed); ¹H NMR δH (400 MHz; CD₃OD) 8.48 (1H, s, H-2α), 8.17 (1H, s, H-8α), 7.32-7.30 (6H, m, Ar-H), 7.19-7.13 (4H, Ar-H), 6.32 (1H, d, J 6.6, H-1'), 5.57 (1H, m, H-2'), 4.95-4.81 (5H, m, 2 x OCH₂Ph, H-3'), 4.35 (1H, m, H-4'), 3.89 (1H, dd, J 12.5, 2.7, H-5's), 3.85 (1H, dd, J 12.5, 2.5, H-5's) and 1.16-1.10 (28H, m, 4 x CH(CH₃)₂); ¹³C NMR δC (100 MHz; CD₃OD) 153.2, 148.8 (C), 148.1 (CH), 141.9 (CH), 135.3 (2 x C), 128.6, 128.5, 127.79 127.75, 127.71, 127.69 (CH), 119.5 (C), 87.9, 87.0, 78.6, 72.3 (CH), 70.1, 70.0, 61.5 (CH₂), 17.0 (8 x CH₃), 13.9 (2 x CH) and 13.6 (2 x CH); ³¹P NMR δₚ (109 MHz; CD₃OD) -1.16. m/z [FAB⁺] 788.4 (M⁺, 100%) [found 788.3276 C₃₆H₅₃N₅O₈PSi₂⁺ requires 788.3276].
2'-(Dibenzyloxyphosphoryl)-3'-O-(3-hydroxy-1,1,3,3-tetraisopropylidisiloxane-1-yl)-5'-H-phosphonate-adenosine

A solution of imidazole (69 mg, 1.01 mmol), PCl₃ (142 µL, 1.62 mmol) and Et₃N (300 µL, 2.16 mmol) in THF (8 mL) was stirred at 0°C for fifteen minutes after which time the 5' alcohol 94 (425 mg, 0.54 mmol) in THF (12 mL) was added in one portion. The reaction was warmed to room temperature and stirred for fifteen minutes, quenched using 1 M aq. triethylammonium bicarbonate (TEAB) (20 mL) and stirred for a further fifteen minutes before the addition of CH₂Cl₂ (20 mL). The organic phase was washed with equal portions of 1 M aq. TEAB (1 x 20 mL), ice water (3 x 20 mL) and 1 M aq. TEAB (3 x 40 mL). The combined aqueous layers were extracted with CH₂Cl₂ (5 x 20 mL). The combined organic layers were dried (MgSO₄), filtered and evaporated to dryness. The oil was then subject to flash chromatography on silica gel eluting with a gradient of EtOAc to EtOAc:CH₃OH:H₂O (10:2:1). The desired fractions were collected and evaporated to dryness to afford the H-phosphonate salt 103 as a white foam (358 mg, 70%).

¹H NMR δH (270 MHz; CDCl₃) 8.69 (1H, s, H-2a d), 8.08 (1H, s, H-8a d), 7.18-7.04 (10H, m, H-Ar), 6.72 (1H, d, J₉H 630, P-H), 6.33 (1H, d, J 5.7, H-1'), 5.45 (1H, m, H-2'), 4.83-4.74 (4H, m, CH₂Ar), 4.73-4.62 (2H, m, H-3', H-4'), 3.96 (2H, m, H-5') and 1.04-0.89 (28H, m, 4 x (CH(CH₃)₂)). ¹³C NMR δC (100 MHz; CDCl₃) 155.4, 150.0 (C), 149.8 (CH), 140.0 (CH), 135.1, 135.0 (C), 128.6, 127.7 (CH), 118.8 (C), 85.1, 79.1, 71.5 (CH) (one carbon under CHCl₃ peak), 69.9, 69.8, 29.9 (CH₂), 17.53 (4 x CH₃), 17.47 (3 x CH₃), 17.39 (CH₃), 13.8 (CH), 13.7 (2 x CH) and 13.5 (CH). 8 aromatic CH occluded; ³¹P NMR δP (109 MHz; CDCl₃) 6.78 and -0.90; m/z [FAB'] 849.9 (M', 100 %) [found 850.2856 C₃₆H₅₆N₅O₁₁P₂Si₂ requires 850.2834].
2'-{(Dibenzyloxyphosphoryl)-3'-O-(3-hydroxy-1,1,3,3-tetraisopropyl disiloxane-1-yl)-5'-phosphate-adenosine

\[ \text{N,O-Bis(trimethylsilyl)acetamide (500 \mu L, 2.0 mmol) was added to a solution of } H\text{-phosphonate 29 (160 mg, 0.17 mmol) in CDCl}_3 (2 \text{ mL}) \text{ and allowed to react for one hour after which time } ^{31}\text{P NMR showed the presence of a new peak at } \delta 116 \text{ ppm. (1S)-(+)\text{-10-(Camphorsulphonyl)oxaziridine 106 (135 mg, 0.59) was added and the oxidation monitored by } ^{31}\text{P NMR (} -17 \text{ ppm). After fifteen minutes the solution was evaporated to dryness and the white precipitate re-suspended in a 1:1 mixture of CH}_3\text{OH:CHCl}_3 (1 \text{ mL}) \text{ with two drops of D}_2\text{O. The reaction was stirred for two hours and monitored by } ^{31}\text{P NMR for the phosphate peak (1.5 ppm). It was then evaporated to dryness and subject to flash chromatography on silica gel eluting with a gradient of EtOAc to EtOAc:CH}_3\text{OH:H}_2\text{O (8:2:1). The desired fractions were collected and evaporated to dryness to afford the phosphate 104 as a white foam (73 mg, 50 %).} \]

\[ ^{1}\text{H NMR } (270 \text{ MHz; CH}_3\text{OD}) \text{ 8.70 (1H, s, H-2aD), 8.14 (1H, s, H-8aD), 7.31-7.04} \text{ (10H, m, H-Ar), 6.43 (1H, d, J 7.4, H-1'\text{), 5.43 (1H, m, H-2'\text{), 4.94-4.90 (4H, m, CH}_2\text{Ar), 4.83-4.64 (2H, m, H-3', H-4'), 4.13 (2H, m, H-5') and 1.09-0.88 (28H, m, 4 x (CH(CH}_3\text{)}_2))}; \text{ }^{13}\text{C NMR } \delta \text{C (67.5 MHz; CDCl}_3) 157.0 \text{ (C), 153.6 \text{ (CH), 151.2 \text{ (C), 141.2 \text{ (CH), 136.4, 136.3 \text{ (C), 129.7 \text{ (4 x CH), 129.6 \text{ (4 x CH), 128.9 \text{ (2 x CH), 119.9 \text{ (C), 87.6, 86.1, 80.2, 74.3 \text{ (CH), 71.1, 71.0, 65.9 \text{ (CH}_2) \text{, 18.1, 18.0 \text{ (CH}_3) \text{, 17.92 \text{ (2 x CH}_3) \text{, 17.89 \text{ (4 x CH}_3) \text{, 14.8 \text{ (2 x CH), 14.5, 14.0 \text{ (CH); } ^{31}\text{P NMR } \delta \text{p (109 MHz; CDCl}_3) 1.2 \text{ and } -0.90. m/z [FAB'] 869.7 (M', 65 \text{ %).} \]
7.6 Synthesis of NADP and NAADP

Nicotinamide adenine dinucleotide 2'-(bisbenzyloxy) phosphate

Carbonyl diimidazole (43 mg, 0.26 mmol) and Et₃N (14 µL, 0.096 mmol) were added to a solution of β-NMN 21 (30 mg, 0.088 mmol) in DMF (300 µL) and stirred at room temperature under a nitrogen atmosphere for three hours monitoring by ³¹P NMR (−9.5 ppm). A few drops of methanol were then added and the solution was stirred for a further five minutes. It was then evaporated to dryness and co-evaporated with DMF (3 x 100 µL). A solution of bisphosphate adenosine 104 (69 mg, 0.08 mmol) in DMF (500 µL) was added and the resulting mixture was stirred at room temperature for twenty-four hours. The reaction material was evaporated to dryness under reduced pressure, suspended in H₂O (5 mL), extracted with CH₂Cl₂ (3 x 5 mL) and the aqueous phase evaporated to dryness before being used crude in the following reaction.

Crude silylated pyrophosphate 108 (70 mg) was suspended in THF (2.5 mL) and treated with a solution of 1 N TBAF in THF (300 µL) buffered with AcOH (15 µL). This reaction was stirred at 0°C for three hours after which complete conversion to a new peak was observed by ion exchange HPLC (R₁ 11 minutes). The reaction was evaporated to dryness, re-suspended in water (10 mL), extracted three times with EtOAc (3 x 10 mL) and loaded onto a column of AG-MP1. The compound was then eluted using a gradient of water:150 mM aq. TFA and the fractions were analysed by ion exchange HPLC (method A). The desired fractions were combined and
lyophilised from D$_2$O to afford monobenzyl NADP 109 as a white hygroscopic powder (11 mg, 14 % from starting adenosine 104).

R$_t$ 11 minutes (AG-MP1 water:150 mM aq. TFA); $^1$H NMR $\delta$H (400 MHz; D$_2$O) 9.26 (1H, s, H-2$_{nic}$), 9.10 (1H, d, J 6.3, H-6$_{nic}$), 8.76 (1H, d, J 8.2, H-4$_{nic}$), 8.37 (1H, s, H-2'$_{ad}$), 8.12 (1H, dd, J 8.2, 6.3, H-5$_{nic}$), 8.01 (1H, s, H-8'$_{ad}$), 7.12-7.09 (3H, m, H-Ar), 6.83-6.81 (2H, m, H-Ar), 6.03 (1H, d, J 6.6, H-1), 5.99 (1H, d, J 5.4, H-1'), 5.11-5.05 (1H, m, H-2'), 4.73 (2H, s, CH$_2$Ar), 4.46-4.43 (2H, m, H-4' and H-2), 4.31 (1H, m, H-3) and 4.23-4.05 (5H, m, H-4, H-5, H-5'). H-3' under HDO peak; $^{13}$C NMR $\delta$C (100 MHz; D$_2$O) 164.5, 149.3 148.7 (C), 146.2, 144.5, 142.9, 142.7, 140.0 (CH), 136.8, 134.0 (C), 128.84, 128.77, 128.44, 128.41, 127.9, 125.9 (CH), 118.3 (C), 100.1, 87.3, 85.4, 84.5, 77.7, 77.1, 74.0 (CH), 70.9 (CH$_2$), 70.5 (CH), 66.7, and 65.6 (CH$_2$); $^{31}$P NMR $\delta$P (109 MHz; D$_2$O) -0.04 and -11.6.

**Nicotinamide adenine dinucleotide phosphate (NADP)**

Monobenzyl NADP 109 (11mg, 0.013 mmol) was suspended in a degassed mixture of H$_2$O:CH$_3$OH (3:1) (2 mL). Cyclohexadiene (100 µL, 1.04 mmol) and 10 % Pd/C (10 mg) were added and the reaction was stirred for two hours at room temperature. The palladium was filtered through a membrane filter, washed with water, applied to a column of AG-MP1 and eluted using a gradient of water:150 mM aq. TFA. Fractions were analysed by ion exchange HPLC and the appropriate fractions (R$_t$ 11 minutes) were combined and lyophilised from D$_2$O to afford NADP 7 as a white hygroscopic powder (3 mg, 31 %).

R$_t$ 11 minutes (AG-MP1 water:150 mM aq. TFA); $^1$H NMR $\delta$H (400 MHz; D$_2$O) 9.30 (1H, s, H-2$_{nic}$), 9.14 (1H, d, J 5.4, H-6$_{nic}$), 8.81 (1H, d, J 8.2, H-4$_{nic}$), 8.46 (1H, s, H-
To a solution of NADP 7 (5.0 mg, 0.007 mmol) in a 100 mM AcOH-NaOH buffer (pH 4, 1.25 mL) was added nicotinic acid (15 mg, 0.12 mmol) and crude ADP ribosyl cyclase enzyme (7 μL). It was stirred at room temperature for six hours and monitored by ion exchange HPLC. Once complete the reaction was applied to an ion exchange column of AG-MP1 and purified using a water-150 mM aq. TFA gradient. The desired fractions were combined, evaporated to dryness, co-evaporated from D$_2$O and lyophilised from D$_2$O to afford NAADP 3 as a white hygroscopic powder (3 mg, 60%).

1H NMR δH (400 MHz; D$_2$O) 9.32 (1H, s, H-2$_{nic}$), 9.18 (1H, d, J 5.9, H-6$_{nic}$), 8.87 (1H, d, J 8.1, H-4$_{nic}$), 8.45 (1H, s, H-2′$_{ad}$), 8.28 (1H, s, H-8′$_{ad}$), 8.13 (1H, m, H-5$_{nic}$), 6.14 (1H, d, J 5.5, H-1), 6.03 (1H, d, J 5.1, H-1′), 4.98 (1H, m, H-2′), 4.51 (1H, m, H-3′), 4.44 (2H, br s, H-4′) 4.41 (1H, m, H-2), 4.32 (1H, m, H-3), 4.27-4.10 (5H, m, H-4, H-5, H-5′); $^{13}$C NMR δC (105 MHz; D$_2$O) 165.5 (COOH), 149.8 (C$_A$4) 148.3 (C$_A$6), 147.0 (CH$_A$2), 144.6 (CH$_N$6), 142.8 (CH$_N$4), 141.8 (CH$_A$8, CH$_N$2), 133.8 (C$_N$3), 128.6 (CH$_N$5), 118.4 (C$_A$5), 99.8 (CH$_n$1′), 86.8 (CH$_A$4′), 83.9 (CH$_A$1′), 77.4 (CH$_N$2′), 76.8 (CH$_A$2′), 73.9 (CH$_N$4′), 70.5
(CH₃3'), 69.9 (CH₂3'), 65.2, and 64.8 (CH₂); ³¹P NMR δₚ (109 MHz; D₂O) 1.47 and
-9.67; m/z [FAB⁺] 744.8 (M⁺, 50 %) [found 745.0658 C₂₁H₂₈N₆O₁₈P₃⁺ requires
745.0673].

7.7 Synthesis of an NAADP analogue
1-Cyano-2,3,5-tri-O-benzoyl-β-D-ribose⁷⁸

![Chemical Structure]

To a stirred solution of 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranose 122 (5.0 g,
9.9 mmol) in CH₂Cl₂ under a nitrogen atmosphere (13 mL) was added TMSCN (2.7
mL, 20.3 mmol). The reaction mixture was treated with one portion of tin (IV)
chloride (1M in CH₂Cl₂) (11.9 mL, 10.2 mmol). The darkening solution was stirred
for two minutes then poured into aq. saturated NaHCO₃ (100 mL) and stirred for five
minutes. CH₂Cl₂ (125 mL) was added and the emulsion filtered through a celite pad.
The CH₂Cl₂ layer was separated and the aqueous layer further extracted with CH₂Cl₂
(3 x 20 mL). The combined organics were dried (Na₂SO₄), filtered and evaporated to
dryness to afford the nitrile 116 as a light orange syrup. A portion was removed and
subject to flash chromatography on silica gel using a gradient of hexane-EtOAc as
eluent. The required fractions were collected and evaporated to dryness to afford the
nitrile 116 as a white foam.

Rᵥ 0.41 (hexane:EtOAc 8:2); mp 59-60 °C (from hexane) (lit.⁷⁸ 58 °C); νₓₙₓ (KBr)/cm⁻¹
1 2956 (Ar-H), 1728 (C=O) and 1268 (C-O); ¹H NMR δₓ (400 MHz; CDCl₃) 8.04-
8.07 (2H, m, Ar-H), 7.85-7.91 (4H, m, Ar-H), 7.48-7.55 (3H, m, Ar-H), 7.29-7.42
(6H, m, Ar-H), 5.94 (1H, dd, J 5.4, 4.4, H-2), 5.79 (1H, t, J 5.4, H-3), 4.92 (1H, d, J
4.4, H-1), 4.66 (1H, dd, J 10.2, 5.4, H-5α), 4.65 (1H, m, H-4) and 4.54 (1H, m, H-5β);
¹³C NMR δₓ (100 MHz; CDCl₃) 166.2, 165.1, 164.9 (C=O), 134.2, 134.0, 133.6,
130.02, 130.00, 129.9 (CH), 129.3 (C), 128.9, 128.8, 128.7 (CH), 128.5, 128.3 (C
115.9 (C), 81.2, 74.7, 72.1, 69.7 (CH) and 63.5 (CH₂), 6 aromatic CH and 1 aromatic
C occluded; m/z [FAB⁺] 472.0 (M⁺, 30 %) [found 472.1394 C₂₇H₂₂NO₇ requires
472.1396].

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Methyl-2-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl) thiazoline-4-carboxylate

Cysteine methyl ester (2.5 g, 14.8 mmol) and Et₃N (2.1 mL, 14.8 mmol) were added to a solution of the crude nitrile 116 (5.0 g, 10.6 mmol) in CH₃OH (200 mL) and stirred under nitrogen at room temperature. After three hours the solution was evaporated to dryness, dissolved in CH₂Cl₂ (20 mL), washed twice with equal volumes of water (2 x 20 mL), 5% aq. NaHCO₃ (2 x 20 mL) and brine (2 x 20 mL). The organic layers were combined, dried (Na₂SO₄), filtered and evaporated to dryness under reduced pressure to afford the thiazoline 123 as an equal mixture of diastereoisomers. A portion was removed and subject to flash chromatography on silica gel using a gradient of hexane-EtOAc as eluent. The required fractions were collected and evaporated to dryness to afford the nitrile as a white foam. 

Rf 0.36 (hexane:EtOAc 7:3); mp 60-63 °C (from hexane); ¹H NMR δH (400 MHz; CDCl₃) 8.13-8.10 (4H, m, H-Ar), 8.00-7.90 (8H, m, H-Ar, H-Ar*), 7.59-7.52 (6H, m, H-Ar, H-Ar*), 7.46-7.33 (12H, m, H-Ar, H-Ar*), 5.91 (1H, t, J 5.1, H-2'), 5.89 (1H, t, J 5.1, H-2'), 5.83 (1H, t, J 5.1, H-3'), 5.82 (1H, t, J 5.1, H-3*), 5.23 (1H, m, H-1'), 5.23 (1H, m, H-1'), 5.18 (1H, t, J 9.4, H-4), 5.14 (1H, t, J 9.4, H-4*), 4.80 (1H, dd, J 10.0, 3.5, H-5'a), 4.77 (1H, dd, J 10.0, 3.5, H-5'a*), 4.69 (2H, m, H-4', H-4*), 4.61 (1H, dd, J 10.0, 4.3, H-5'b), 4.58 (1H, dd, J 10.0, 4.3, H-5'b*), 3.79 (3H, s, CH₃), 3.70 (3H, s, CH₃*), 3.59 (1H, dd, J 11.5, 9.3, H-5a), 3.57 (1H, dd, J 11.5, 9.3, H-5a*), 3.52 (1H, dd, J 11.5, 9.3, H-5b) and 3.46 (1H, dd, J 11.5, 9.3, H-5b*); ¹³C NMR δC (100 MHz; CDCl₃) 174.0, 173.9, 171.0, 170.6, 166.22, 166.18 (C, C*), 165.3 (C and C*), 165.14, 165.09 (C, C*), 133.6 (CH and CH*), 133.5 (CH and CH*), 133.2 (CH and CH*), 129.9, 129.8 (6 x CH, 6 x CH*), 129.63, 129.59, 129.0, 128.9, 128.7 (3 x C, 3 x C*), 128.5 (6 x CH, 6 x CH*), 80.8, 80.6, 78.14, 78.10, 74.9, 74.8, 72.54, 72.47 (CH, CH*), 64.0, 63.9 (CH₂, CH₂*), 60.4 (CH and CH*), 52.83, 52.76 (CH₃, CH₃*), 34.6 and 34.4 (CH₂, CH₂*); m/z [FAB⁺] 590.2 (M⁺, 35%).
Methyl 2-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl)thiazole-4-carboxylate

To a solution of crude thiazoline 123 (4.7 g, 8.0 mmol) in CH$_2$Cl$_2$ (250 mL) was added DBU (16 mmol, 2.4 mL). This was cooled to 0°C and BrCCl$_3$ (0.95 mL, 9 mmol) was added dropwise, stirred overnight and concentrated under vacuum. The solution was washed three times with aq. saturated NH$_4$Cl (3 x 20 mL) and the aqueous phase extracted twice with EtOAc (2 x 20 mL). The combined organics were dried (Na$_2$SO$_4$), filtered and evaporated to dryness under vacuum. The residue was subject to flash chromatography on silica gel using a gradient of hexane-EtOAc (9:1 to 7:3) as eluent. The required fractions were collected and evaporated to dryness to afford the thiazole 121 as a light yellow foam (3.6 g, 61% over three steps).

R$_f$ 0.48 (hexane:EtOAc, 6:4); mp 61 °C (from hexane); [$\alpha$]$^{25}_{D}$ $-$43 (c 1 in CHCl$_3$; ν$_{max}$ (KBr)/cm$^{-1}$ 2962 (Ar-H), 1726 (C=O) and 1269 (C-O); $^1$H NMR $\delta_H$ (400 MHz; CDCl$_3$) 8.15 (1H, s, H-5), 8.09-8.07 (2H, m, H-Ar), 8.00-7.97 (2H, m, H-Ar), 7.92-7.89 (2H, m, H-Ar), 7.58-7.51 (3H, m, H-Ar), 7.45-7.3 (6H, m, Ar-H), 5.91-5.88 (2H, m, H-2', H-3'), 5.75 (1H, d, J 4.7, H-1'), 4.89 (1H, dd, J 12.1, 3.1, H-5'a), 4.78-4.75 (1H, m, H-4') 4.61 (1H, dd, J 12.1, 3.9, H-5''), and 3.91 (3H, s, CH$_3$); $^{13}$C NMR $\delta_C$ (100 MHz; CDCl$_3$) 169.6, 166.2, 165.3, 165.2, 161.6, 147.3 (C), 133.72, 133.68, 133.5, 130.1, 129.92, 129.90 (CH), 129.6, 129.0, 128.9 (C), 128.8, 128.7, 128.6, 80.9, 80.8, 76.8, 72.5 (CH), 64.0 (CH$_2$) and 52.8 (CH$_3$), 7 aromatic CH occluded; m/z [FAB $^+$] 588.1 (M$^+$, 56%) [found 588.1342 C$_{31}$H$_{26}$NO$_9$S requires 588.1328].
Methyl 2-(β-D-ribofuranosyl)thiazole-4-carboxylate

\[ \text{Methyl 2-(\beta-D-ribofuranosyl)thiazole-4-carboxylate} \]

\[
\begin{align*}
\text{CH}_3\text{ONa}^+ & \text{ (92 mg, 0.017 mmol) was added to a solution of thiazole 121 (100 mg, 0.17 mmol) in \text{CH}_3\text{OH (3 mL) and stirred overnight at room temperature then quenched with Dowex 50WX 4-100. The mixture was filtered, evaporated to dryness subject to flash chromatography on silica gel using a gradient \text{CH}_2\text{Cl}_2 to \text{CH}_2\text{Cl}_2: \text{CH}_3\text{OH (100 to 97:3) as eluent to afford the methyl carboxylate 124 as a white solid (37 mg, 80 \%)}.} \\
\text{R}_f & \text{ 0.46 (CH}_2\text{Cl}_2: \text{CH}_3\text{OH, 9:1); mp 119-121 °C (from \text{CH}_2\text{Cl}_2); } [\alpha]_{D}^{25} \text{ 0 (c 1 in \text{CH}_3\text{OH}); } \nu_{\text{max}} \text{ (KBr disk)/cm}^{-1} \text{ 3369 (O-H), 1726 (C=O) and 1237 (C-O); } ^1\text{H NMR } \delta_{\text{H}} \text{ (400 MHz; CD}_3\text{OD) 8.20 (1H, s, H-5), 4.95 (1H, d, } J 5.5, \text{ H-1', } 4.10 \text{ (1H, m, H-4'), 3.98-3.94 (2H, m, H-2', H-3'), 3.72 (3H, s, CH}_3\text{), 3.68 (1H, dd, } J 12.5, 2.3 \text{ H-5''a) and 3.56 (1H, dd, } J 12.5, 18.5 \text{, H-5''b); } ^{13}\text{C NMR } \delta \text{ (100 MHz; CD}_3\text{OD) 172.2, 163.1, 145.7 (C), 129.8, 84.7, 81.8, 76.8, 71.2 (CH), 61.8 (CH}_2\text{) and 53.0 (CH}_3\text{); } m/z \text{ [FAB'] 276.1 (M', 100 %)] found 276.0538 C}_{10}\text{H}_{14}\text{N}_2\text{O}_6\text{S requires 276.0542}.} \\
\end{align*}
\]

2-(β-D-Ribofuranosyl)thiazole-4-carboxamide (Tiazofurin)

\[ \text{2-(\beta-D-Ribofuranosyl)thiazole-4-carboxamide (Tiazofurin)} \]

\[
\begin{align*}
\text{To a stirred solution of freshly prepared saturated methanolic ammonia (5 mL) at 0°C was added the methyl carboxylate 121 (40 mg, 0.68 mmol). The reaction was stirred at room temperature overnight in a glass pressure tube, then evaporated to dryness, re-suspended in methanolic ammonia (5 mL), and stirred for a further twenty-four hours. The solvent was evaporated, the residue dissolved in water (10 mL) and washed with \text{EtOAc (5 x 20 mL). The aqueous phase was concentrated under reduced pressure and} \\
\end{align*}
\]

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lyophilised from D$_2$O to afford tiazofurin 111 as a yellowish waxy solid (152 mg, 86%).

mp 145-147 °C (from D$_2$O) (lit.75, 143 °C); [α]$^D_{25}$ −7.5 (c 1 in H$_2$O); $\nu_{\text{max}}$ (film)/cm$^{-1}$ 3390 (O-H) and 1667 (C=O); $^1$H NMR $\delta_{\text{H}}$ (400 MHz; D$_2$O) 8.02 (1H, s, H-5), 4.98 (1H, d, J 5.1, H-1'), 4.16 (1H, dd, J 5.5, 3.9, H-4'), 4.03-3.99 (2H, m, H-2', H-3'), 3.70 (1H, dd, J 12.5, 3.9, H-5'a) and 3.59 (1H, dd, J 12.5, 5.5, H-5'b); $^{13}$C NMR $\delta_{\text{C}}$ (100 MHz; D$_2$O) 171.5, 165.4, 148.3 (C), 129.2, 84.9, 81.7, 76.7, 71.4 (CH) and 61.9 (CH$_2$); $m/z$ [FAB$^+$] 261.1 (M$^+$, 100 %) [found 261.0543 C$_9$H$_{13}$N$_2$O$_5$S requires 261.0545].

2-(β-D-Ribofuranosyl)thiazole-4-carboxylic acid

To a stirred solution of methyl carboxylate 121 (10 mg, 0.17 mmol) in dioxane (3 mL) was added 1 M NaOH (3 mL) and stirred overnight. The reaction was acidified using Dowex resin (50WX 4-100), filtered, washed with water and extracted three times with EtOAc (3 x 5 mL). The aqueous phase was concentrated under reduced pressure and lyophilised from D$_2$O to afford the carboxylic acid 113 as a white hygroscopic solid (38 mg, 86 %).

$[\alpha]^D_{25}$ −10 (c 1 in H$_2$O); $\nu_{\text{max}}$ (film)/cm$^{-1}$ 3390 (O-H) 1713 (C=O); $^1$H NMR $\delta_{\text{H}}$ (400 MHz; D$_2$O) 8.02 (1H, s, H-5), 5.01 (1H, d, J 5.1, H-1'), 4.18 (1H, m, H-4'), 4.03-4.01 (2H, m, H-2', H-3'), 3.72 (1H, dd, J 12.5, 3.1, H-5'a) and 3.61 (1H, dd, J 12.5, 5.1, H-5'b); $^{13}$C NMR $\delta_{\text{C}}$ (100 MHz; D$_2$O) 172.1, 163.8, 146.1 (C), 129.9, 84.6, 81.9, 76.8, 71.2 (CH) and 61.9 (CH$_2$). $m/z$ [FAB$^+$] 261.9 (M$^+$, 100 %) [found 262.0398 C$_9$H$_{13}$NO$_5$S requires 262.0385].
Methyl-2-(5'-phosphoryl-β-D-ribofuranosyl)thiazole-4-carboxylate

POCl₃ (20 µL, 0.22 mmol) was added to a stirred solution of methyl carboxylate 124 (50 mg, 0.18 mmol) in triethylphosphate (0.75 mL) under a nitrogen atmosphere at 0°C. The reaction was monitored by using an ion exchange HPLC (method A) and after ninety minutes the reaction was quenched using H₂O (5 mL) and stirred for 5 minutes. The excess triethylphosphate was extracted five times with ethyl acetate (5 x 15 mL) and the aqueous phase loaded onto ion exchange gradifac column (AG-MP1) eluted with a gradient of 150 mM aq. TFA. Fractions were analysed by ion exchange HPLC (method A), desired fractions collected, evaporated to dryness and lyophilised from D₂O to afford the phosphate 127 as a light yellow hygroscopic gum (46 mg, 72%).

Rt 14 minutes (AG-MP1 water:150 mM aq. TFA); ¹H NMR δH (400 MHz; D₂O) 8.13 (1H, s, H-5), 4.88 (1H, d, J 5.1, H-1'), 4.06 (1H, t, J 5.1, H-4'), 4.01-3.98 (2H, m, H-2', H-3'), 3.93-3.80 (2H, m, H-5') and 3.63 (3H, s, CH₃); ¹³C NMR δC (100 MHz; D₂O) 172.4, 163.0, 145.4 (C), 130.0, 83.3, 82.0, 76.8, 71.3 (CH), 65.4 (CH₂) and 53.0 (CH₃); ³¹P NMR δP (162 MHz; D₂O) 0.97.

2-(5'-Phosphoryl-β-D-ribofuranosyl)thiazole-4-carboxylic acid

POCl₃ (70 µL, 0.75 mmol) was added to a stirred solution of methyl carboxylate 124 (180 mg, 0.67 mmol) in triethylphosphate (3.0 mL) under a nitrogen atmosphere at 0°C. The reaction was monitored by using an ion exchange HPLC (method A) and after 1.5 hours the reaction was quenched using H₂O (5 mL) and stirred for 5 minutes. The excess triethylphosphate was extracted five times with ethyl acetate (5 x 15 mL)
and the pH of the aqueous phase adjusted to 10 with 1 M NaOH and stirred overnight then loaded onto ion exchange column (AG-MP1) and eluted using a gradient of TFA. Fractions were analysed by ion exchange HPLC (method A), desired fractions collected, evaporated to dryness and lyophilised from D$_2$O to afford the phosphate 128 as a light yellow hygroscopic solid (187 mg, 82 %).

R$_T$ 17 minutes (AG-MP1 water:150 mM aq. TFA); $^1$H NMR $\delta_H$ (400 MHz; D$_2$O) 8.14 (1H, s, H-5), 4.92 (1H, d, J 5.1, H-1'), 4.09 (1H, t, J 4.9, H-4'), 4.07-4.01 (2H, m, H-2', H-3') and 4.01-3.88 (2H, m, H-5'); $^{13}$C NMR $\delta_C$ (100 MHz; D$_2$O) 172.5, 163.7, 145.7 (C), 130.2, 82.9, 81.9, 76.7, 71.0 (CH) and 65.5 (CH$_2$); $^{31}$P NMR $\delta_P$ (162 MHz; D$_2$O) 0.21.

2-Thiazole-4-carboxylate adenine dinucleotide phosphate

\[
\begin{align*}
\text{Carbonyl diimidazole (88 mg, 0.6 mmol) and Et$_3$N (31 \mu L, 0.22 mmol) were added to a solution of methyl carboxylate phosphate 127 (70 mg, 0.2 mmol) in DMF (500 \mu L) and stirred at room temperature under a nitrogen atmosphere for three hours ($^{31}$P NMR $\delta$–10 ppm). Two drops of CH$_3$OH were added and the solution stirred for further 10 minutes. The reaction was evaporated to dryness, co-evaporated with DMF (3 x 100 \mu L), a solution of diphosphate adenosine 74 (151 mg, 0.19 mmol) in DMF (500 \mu L) added and the reaction stirred overnight. The solution was applied to a column of Q-Sepharose and eluted with a gradient of water- 2 M aq. TEAB. The desired fractions were analysed by ion exchange HPLC, collected and evaporated to dryness. The product was then used crude in the following reaction.}
\end{align*}
\]
To a crude solution of protected pyrophosphate 131 (60 mg) in CH$_3$OH (1.5 mL) was added 1 M NaOH (1.5 mL) and stirred overnight. $^{31}$P NMR showed a new peak at 4.2 ppm corresponding to a free phosphate. The reaction was neutralised to pH 7, loaded onto a Q-Sepharose column and eluted with a gradient of water- aq. 2 M TEAB. The desired fractions were analysed by HPLC, collected and evaporated to dryness. The product was then used crude in the following reaction.

HF/pyridine (7:3 ν/ν) (25 μL, 0.96 mmol) was added to a solution of crude silyl protected pyrophosphate 132 (23 mg) in pyridine (1 mL) and stirred for 20 minutes then neutralised with aq. saturated NaHCO$_3$. The solution was evaporated to dryness, re-suspended in H$_2$O, applied to a column of AG-MP1 and eluted using a gradient of water:150 mM aq. TFA. The desired fractions were analysed, collected, evaporated to dryness and lyophilised from D$_2$O to afford TAADP 114 as a white hygroscopic powder (7 mg, 5 % over three steps from starting adenosine 74).

R$_t$ 20 minutes (AG-MP1 water:150 mM aq. TFA); $^1$H NMR $\delta$ (400 MHz; D$_2$O) 8.39 (1H, s, H-2'$_{ad}$), 8.25 (1H, s, H-8'$_{ad}$), 8.09 (1H, s, H-5$_{th}$), 6.12 (1H, d, J 5.5, H-1'), 5.03 (1H, m, H-2'), 4.91 (1H, d, J 5.1, H-1), 4.52 (1H, t, J 3.9, H-3'), 4.29 (1H, m, H-4'), 4.14 (5H, m, H-2, H-3, H-4, H-5') and 4.00 (2H, m, H-5); $^{13}$C NMR $\delta$ (150 MHz; D$_2$O) 172.9, 164.1, 150.0 (C), 148.6, (CH) 145.8 (C), 144.9, 143.2 (CH), 130.5, 118.7 (C), 87.2, 84.3, 83.5, 82.0, 77.3, 77.1, 71.5, 70.3 (CH), 66.0 and 65.7 (CH$_2$); $^{31}$P NMR $\delta$ (109 MHz; D$_2$O) 0.73 and 10.69; m/z [FAB'] 749.0 (M', 100 %) [found 749.0062 C$_{19}$H$_{26}$N$_6$O$_9$P$_3$S requires 749.0081].
Reference List


74. Tiazofurin is the generic name approved by the United States Adopted Name Council.

Appendix
A concise route to tiazofurin

Richard S. Brown, James Dowden,* Christelle Moreau and Barry V. L. Potter

Wolfson Laboratory of Medicinal Chemistry, Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, UK

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Abstract—Successful oxidation of a key thiazoline intermediate allows an efficient synthesis of tiazofurin in four steps from commercially available 1′-acetoxy-2′,3′,5′-tri-0-benzoyl-β-D-ribofuranose. © 2002 Elsevier Science Ltd. All rights reserved.

Tiazofurin 4 is converted in vivo into the active metabolite thiazole-4-carboxamide adenine dinucleotide (TAD), an analogue of NAD that prevents de novo guanine nucleotide synthesis via inhibition of inosine monophosphate dehydrogenase (IMPDH, EC 1.1.1.205). The consequent decrease in cellular GTP and deoxyGTP concentrations interrupts DNA and RNA synthesis in rapidly-dividing tumour cells. Tiazofurin proved effective in reducing the leukaemic cell burden in acute myelogenous leukaemia patients, but was found to be too toxic for general clinical application. Subsequent discovery of two IMPDH isoforms, of which type II is up-regulated in human leukaemia cell lines, has prompted studies to inform the design of isoform selective inhibitors and has renewed interest in tiazofurin and its analogues.

Several routes have been published toward this compound; of these the preparation reported by Ramasamy and co-workers provides tiazofurin, while avoiding the use of hydrogen sulfide gas. These authors reported unsuccessful oxidation of thiazoline 1 with MnO₂, or a range of other conditions, observing elimination of the 2′,3′-benzoate esters to afford thiazole-furan 2 in each case (Scheme 1).

Instead, isopropylidene 3 was converted into the target compound 4 with good reported yields, but the introduction and removal of the isopropylidene protecting group introduced an extra three steps from 1′-acetoxy-2′,3′,5′-tri-0-benzoyl-β-D-ribofuranose 5 (Scheme 2).

We now report the successful oxidation of thiazoline 1 that removes the need for this detour and reduces the synthesis of tiazofurin to a simple four-step procedure.

Thiazoline 1 was prepared by reaction of 1′-cyano-2′,3′,5′-tri-0-benzoyl-β-D-ribofuranosyl with L-cysteine methyl ester in the presence of triethylamine according to an established protocol. Complete epimerisation of the cysteine α-proton was observed by 1H NMR, but this centre will become part of the aromatic thiazole. A number of oxidation conditions, including DDQ in toluene, did not proceed to our satisfaction, but we were pleased to observe clean conversion of the thiazoline 1 in the presence of bromotrichloromethane and DBU. Thiazole 6 was thus prepared from commercial thiazoline 1.

Scheme 1. Reagents and conditions: (a) MnO₂/benzene/reflux.

Scheme 2. Reagents and conditions: (a) cysteine ethyl ester HCl/Et₃N; (b) MnO₂/benzene/reflux; (c) 90% TFA; (d) NH₃/MeOH.

* Corresponding author. E-mail: j.dowden@bath.ac.uk

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Ester aminolysis and global deprotection were effected in the presence of sensitive ribose 2',3',5'-tribenzoate in 86% yield. Dissolution of the concentrated crude reaction mixture into water and extraction of all contaminants into ethyl acetate proved beneficial this discovery is to improve the efficiency of tiazofurin synthesis in the absence of hydrogen sulfide affording a productive four-step protocol.

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References

1. Tiazofurin is the generic name approved by the United States Adopted Name Council.
15. Preparation of methyl 2-(2',3',5'-tri-O-benzoyl-ß-D-ribofuranosyl)thiazole-4-carboxylate 1 (4.7 g, 8 mmol) in CH2Cl2 (250 mL). The solution was cooled to 0°C and BrCCl3 (1.9 g, 9 mmol, 1.0 mL) was added dropwise and the resulting mixture stirred overnight. The reaction mixture was then concentrated, dissolved into ethyl acetate and the solution washed 3x with satd aq. NH4Cl. The organic layer was dried (Na2SO4) filtered, concentrated and subject to column chromatography on silica gel using a gradient of hexane:ethyl acetate to afford 6 as a light yellow foam. Yield 3.6 g, 6.1 mmol, 61% over three steps. \( \phi = -43.3 \) (1, CHCl3); IR (KBr) 1726, 1269, 1095, 710 cm\(^{-1}\); \( ^1\)H NMR (400 MHz CDCl3): \( \delta = 8.15 \) (1H, s, SCH) 8.09–8.07 (2H, m, Ar-H) 8.00–7.97 (2H, m, Ar-H) 7.92–7.87 (2H, m, Ar-H) 7.58–7.51 (3H, m, Ar-H) 7.45–7.33 (6H, m, Ar-H) 5.91–5.88 (3H, m, H-2,3) 5.75 (1H, d, \( J = 4.7 \), H-1) 4.89 (1H, dd, \( J = 12.1, 3.1, 1H, H-5'a) 4.75–4.78 (1H, m, H-4') 4.61 (1H, dd, \( J = 12.1, 3.9, 1H, H-5'b) 3.91 (3H, s, COOCH3); \( ^{13}\)C NMR (100 MHz (DEPT) CDCl3): \( \delta = 169.6(0), 166.2(0), 165.3(0), 165.2(0), 161.6(0), 147.3(0), 133.8(1), 133.7(1), 133.5(1), 130.1(1), 129.92(1), 129.91(1), 129.60(1), 128.98(0), 128.92(0), 128.76(1), 128.71(1), 128.61(1), 80.9(1), 88.0(1), 76.9(1), 72.5(1), 64.0(2), 52.8(3), MS (FAB+ \( m/z \) (M+H)+) 588 (100), MS (FAB+ \( m/z \) (M+H)+) 588 calculated for \( C_{31}H_{25}N_9O_9S: 588.1341 \). Found: 588.1341.
Modulation of intracellular calcium concentration creates signals that control a range of biological pathways. Orchestration of these signals by an array of small molecule second messengers provides the necessary complexity to complement multiple cellular responses, such as activation of the immune system or fertilization. The discovery that nicotinic acid adenine dinucleotide phosphate (NAADP, 1; Figure 1) potently induces intracellular calcium release in the eggs of sea urchins raised its status, from minor contaminant of nicotinamide adenine dinucleotide phosphate (NADP, 2), to an important novel second messenger. Recent studies attest to the higher potency of this molecule than the well-known messengers for calcium release (i.e., inositol trisphosphate and cyclic adenosine diphosphoribose (cADPR)) and many of these extend its actions to a wide range of mammalian cells, including cardiac, skeletal, T cells, smooth muscle, pancreatic acinar, and pancreatic beta cells.

Compound 1 also appears to play an important role in calcium signaling by triggering and coordinating calcium signals evoked by other calcium-mobilizing messengers. Sensitive radio-receptor assays for tissue measurements of this dinucleotide have been developed, but it is clear that further understanding of its biology will draw heavily on the development of selective molecules to probe this novel signaling pathway and characterize its protein components. A chemo-enzymatic route has so far generated 1 and a small number of analogues from 2 by enzyme-mediated exchange of nicotinamide for nicotinic acid. Clearly, the chemistry that can be performed on this sensitive molecule and compatibility with the enzyme limits the range of available analogues. A synthetic route would provide a wider range of tailored derivatives, such as non-hydrolysable analogues, that could not be achieved by established methods.

We are currently exploring a range of strategies to generate chemical tools with which to interrogate this biological system, including the total synthesis of both dinucleotides. Whitesides and co-workers developed a semisynthetic route to p-nicotinamide adenine dinucleotide (NAD) by using NAD pyrophosphorylase immobilized on polyacrylamide gel, but this relied on a battery of enzymes and reagents to generate stoichiometric quantities of adenosine triphosphate (ATP). More recently, Lee et al. reported a practical total chemical synthesis of nicotinamide adenine dinucleotide (NAD) that built upon a much earlier synthesis by Todd and co-workers. Indeed, a number of researchers have developed chemical approaches to NAD derivatives, not least for the preparation of cyclic adenosine 5'-diphosphate ribose. However, the presence of a sensitive 2'-phosphate on a ribose makes the synthesis of 1 or 2 significantly more complex. During early studies Todd and co-workers reported an apparent preparation of 2 inferred from biological evaluation of the complex product mixture but did not successfully isolate the desired coenzyme from the mixture.

Given that this dinucleotide is readily available from commercial sources, it is perhaps not surprising that further developments toward its chemical synthesis are not apparent.
Communications

The potentially fragile nature of 2 and its intermediates encouraged a conservative approach that involves the late construction of the pyrophosphate bond by activation of β-NMN, 4; Scheme 1) and reaction with a suitably decorated adenosine building block. It is known that the 2'-phosphate motif is critical for the activity of 1 and may be involved in its degradation. Structural modifications are likely to be informative and thus we designed a synthesis that was both robust and sufficiently flexible to accommodate future modifications at any position on the NAADP framework.

Compound 4 was generated from tetra-O-acetyl-β-d-ribose. Reaction between adenosine and 1,3-dichloro-1,1,3,3-tetraisopropyl disiloxane in pyridine proceeded without complication and lead to simultaneous tethering of the 3' and 5'-hydroxyls of adenosine, thus permitting unambiguous phosphorylation of the 2'-alcohol. Subsequent elaboration of the 2'-alcohol 5 could be achieved with a range of phosphoramidites, including bis(benzoxyl)-N,N-diisopropylamino-phosphate 6 (Scheme 1), to afford the respective phosphate 7 in high yield after oxidation of the intermediate phosphate with m-chloroperoxybenzoic acid (mCPBA). Imidazolium triflate was used instead of tetrazole to produce a less reactive phosphorylating intermediate that does not react with the purinyl amine, thus saving two steps. Carefully controlled acidic conditions, as previously reported by Scott and co-workers afforded exclusive protodesilylation at the less hindered 5'-position in quantitative yield.

Generally, phosphoramidite chemistry did not proceed in acceptable yields at the 5'-hydroxyl of the precursor, presumably because this position is somewhat crowded. Resorting to the reactive tetrazole-activated phosphoramidites required amine protection and actually led to incorporation of an unwelcome silanol phosphate. It was frustrating that a multitude of protection strategies failed due to unexpected incompatibility with the desired route. Although this chemistry might be optimized to provide acceptable yields of the desired compound, we found that H-phosphonate chemistry offered a very effective alternative. Selective 5'-protodesilylation of bis(benzoxyl)phosphate 7, then treatment of the product with trisimidazolylphosphane, generated in situ from PC13 and imidazole, rapidly furnished 8 in quantitative yield (Scheme 1). Oxidation was best achieved by using conditions reported by Sekine and co-workers in a process monitored by 31P NMR spectroscopy, such that the H-phosphonate triethylammonium salt (δ(31P) = 5.5 ppm) was treated with N,N-(trimethylsilyl)acetamide (BSA) to afford the bis(tri-methylsilyl)phosphate (δ(31P) = −16 ppm) in about 20 min, which
slowly hydrolyzed to give the phosphate 9 (\(\delta^{(31}P) = -10.6 \) ppm) upon addition of D$_2$O.

Successful repetition of the approach to NAD reported by Lee and co-workers\(^{[10]}\) encouraged initial exploration of phosphomimetic activation of the precursor 8, but attempted conversions were not productive. Production and reaction of the \(\beta\)-NMM imidazolide could be monitored by using \(^{31}P\) NMR spectroscopy (\(\delta^{(31}P) = -10.6 \) ppm).

Contrary to other reports,\(^{[10]}\) we found that bisbenzylphosphate precursor 9 appeared to react with good conversion when monitored by \(^{31}P\) NMR spectroscopy and offered an attractive protecting-group strategy (Scheme 1). Both benzyl groups remained intact during this reaction and the resulting product could not be purified by using our preferred ion-exchange chromatography method. Instead, the crude material was treated with 1m TBAF in THF/AcOH to effect simultaneous cleavage of the silanol ether and one of the benzyl protecting groups to yield monobenzyl NADP 10 after ion-exchange chromatography. Although there was no evidence of migration of the 2'-phosphate, the overall yield of around 22% for these two steps is modest, but is comparable to many literature reports as pyrophosphate bond formation and isolation of the resulting product is generally difficult and yields are highly variable. An alternative route that employs a bis(2-cyanoethyl) protected precursor related to 9 gave yields in the range of 60-80% for the pyrophosphate coupling and we are currently exploring improvements of this route to achieve useful conversion. Nonetheless, transfer hydrogenation of 10 yielded material that was identical to commercial preparations of \(\beta\)-NADP 2 in satisfactory (77%) yield.

With a wider objective in mind we chose to convert synthetic NADP 2 into the target NAADP 1 to test its behavior in a relevant biological system. Base exchange to replace nicotinamide with nicotinic acid was achieved by using an excess of the latter and crude Aplysia ADP-ribosyl cyclase \(E.C.3.2.2.5\) prepared from Aplysia ovotestis\(^{[13]}\) to provide NAADP 1 in 63% yield.

The ability of 1 to induce release of Ca$^{2+}$ ions was tested with a cell-free system derived from the eggs of sea urchins. Aliquots of homogenates of sea-urchin eggs comprise vesicles derived from the intracellular stores that sequester calcium when supplemented with an ATP-regenerating system. Concentration-dependent calcium release is observed when this mixture is challenged with second messengers, such as 1, which can be measured by using cuvette-based fluorimetry and the calcium reporter dye fluo-3.\(^{[10]}\) Synthetic NAADP, carefully quantified by using total phosphate assay prior to evaluation,\(^{[10]}\) potently induced calcium release in an identical manner to “authentic” NAADP prepared from commercial NADP (Figure 2). Both preparations display an important characteristic of this signaling pathway that occurs at subthreshold concentrations of NAADP in homogenate of sea-urchin eggs: They both potently deactivate the calcium store in a time dependent manner, so that further challenge with NAADP does not lead to significant calcium release (Figure 3). This property allows competition binding experiments that measure the extent that nonlabeled NAADP displaces subthreshold concentrations of \([^{32}P]\)NAADP (0.2 nm) from the putative receptor (Figure 4).

In summary, the strategy of using 1,1,3,3-tetraisopropyl disiloxane protecting groups offers a robust (70%, five steps from adenosine) route to 2',5'-adenosine diphosphate precursors and the potential to incorporate a range of functionality at these positions. Pyrophosphate formation requires some further development, but affords benzyl protected precursor 10 that, after transfer hydrogenation, leads to the first total chemical synthesis of 2. This flexible route to 2'-phosphate furnished dinucleotides will allow the development of new chemical tools that probe biological systems at the molecular level. Analogues of 1 are of immediate importance for dissecting this novel and important second

Figure 2. Ca$^{2+}$-ion release from homogenate of sea-urchin eggs by 100 nm authentic NAADP (dotted arrows) or 100 nm synthetic NAADP, 1 (solid arrows): Samples diluted to 2.5% in GluIM in the presence of a regenerating system and kept at 17°C with agitation for 3 h to facilitate Ca$^{2+}$-ion uptake into stores. Ca$^{2+}$-ion release determined by an increase of Fluo-3 fluorescence at 526 nm; data expressed as released [Ca$^{2+}$], determined by fluorescence in arbitrary units; R. F. U. = relative fluorescence units.

Figure 3. Inhibition of NAADP-induced Ca$^{2+}$-ion release by authentic NAADP (squares) or synthetic NAADP 1 (triangles) in the homogenate of sea-urchin eggs: Samples diluted to 2.5% in GluIM in the presence of the regenerating system and kept at 17°C, with agitation for 3 h to facilitate Ca$^{2+}$-ion uptake into stores. Ca$^{2+}$-ion release determined by an increase of Fluo-3 fluorescence at 526 nm; data expressed as pmol of Ca$^{2+}$-ions released; n = > 3 for each point.
messenger pathway. Enzymatic conversion into 1 is confirmed both by identical behavior when evaluated for Ca²⁺-release properties against sea-urchin-egg homogenate and spectroscopic characterization. Expansion of the route to include suitably protected beta-nicotinic acid mononucleotide (P-NAMN) towards the first total chemical synthesis of 1 and improved isolated yields of pyrophosphate will be reported in due course.

Experimental Section

2: Benzyl NADP 9 (15 mg, 0.018 mmol), 10% Pd/C (15 mg) and cyclohexadiene (100 µL) in a degassed mixture of H₂O:MeOH (3:1, v/v, 2 mL) were stirred at room temperature under an argon atmosphere and lyophilized overnight to afford 1 as a white powder (2.5 mg, 8171-8178). The crude mixture was purified on an ion-exchange resin (AG MP-1) by using a 150 mM aqueous TFA gradient. Fractions were combined and lyophilized to yield 2 as a white powder (10 mg, 77%): 'H NMR (400 MHz, D₂O): δ = 7.72 (1H, H₂), 7.64 (1H, H₂), 7.41 (1H, H₂), 7.20-7.10 (m, 3H, H₃, H₄, H₅); 13C NMR (100 MHz, D₂O): δ = 165.4 (CO₂H), 149.8 (C₄), 148.3 (C₆), 147.0 (CH₂), 144.6 (CH₆), 142.7 (CH₇), 142.5(CH₈), 141.8 (CH₉), 133.8 (C₇), 128.6 (CH₅), 118.4 (C₅), 99.7 (CH₁), 86.8 (CH₄), 85.9 (CH₁'), 77.4 (CH₂'), 76.8 (CH₂'), 73.6 (CH₄'), 70.3 (CH₃'), 69.9 (CH₃), 65.1 and 64.8 ppm (2×CH₃); 3¹PNMR (162 MHz, D₂O): δ = 1.47 and -9.40 to -9.80 ppm (br m); MS [FAB⁺]: 744.8 [M⁺-H]; found 745.0658 [M⁺-H] C₇H₅N₃O₅P requires 745.0673.

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