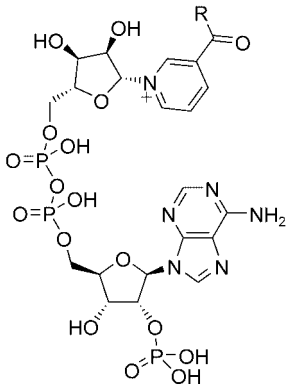

1 NAADP; R = OH
2 NADP; R = NH_2

Figure 1. Compounds **1** and **2**.

Natural Products Synthesis

Chemical Synthesis of the Second Messenger Nicotinic Acid Adenine Dinucleotide Phosphate by Total Synthesis of Nicotinamide Adenine Dinucleotide Phosphate**

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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.[1] The discovery that nicotinic acid adenine dinucleotide phosphate (NAADP, **1**; Figure 1) potently induces intracellular calcium release in the eggs of sea urchins[2] raised its status, from minor contaminant of nicotinamide adenine dinucleotide phosphate (NADP, **2**), to an important novel second messenger.[3] 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,[4a] skeletal,[4b] T cells,[4c] smooth muscle,[4d] pancreatic acinar[4e] and pancreatic beta cells.[3a,4f,4g]

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,[5] 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 β-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).[6a] More recently, Lee et al.[6b] reported a practical total chemical synthesis of nicotinamide adenine dinucleotide (NAD) that built upon a much earlier synthesis by Todd and co-workers.[7] Indeed, a number of researchers have developed chemical approaches to NAD derivatives, not least for the preparation of cyclic adenosine 5′-diphosphate ribose.[8] 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.[7b] 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

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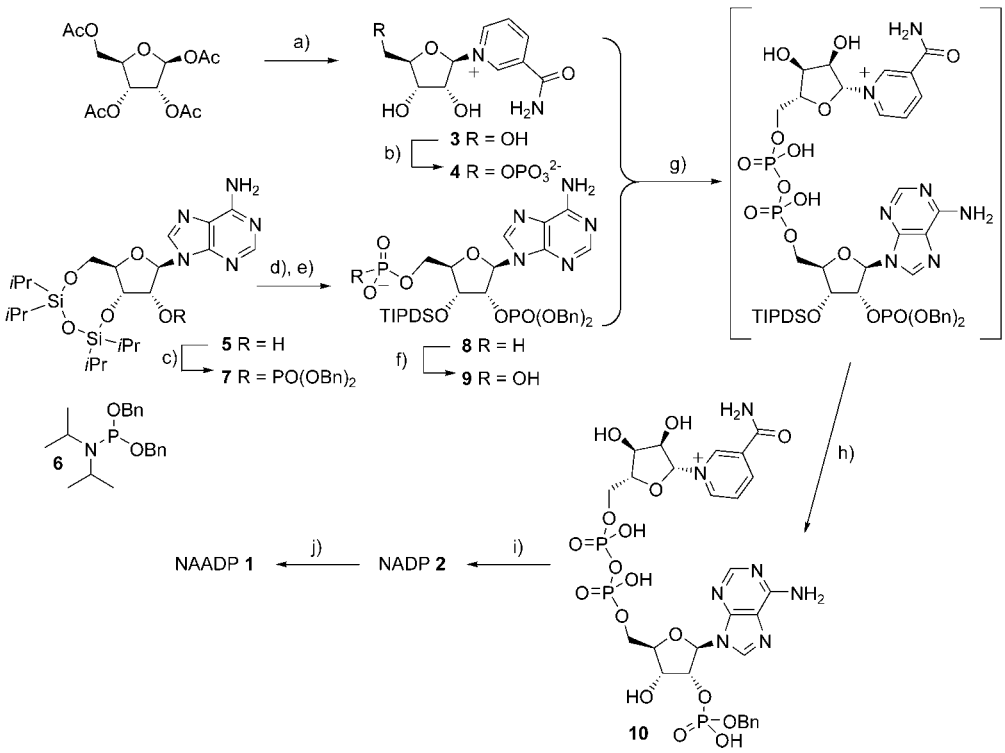
in the general literature. To the best of our knowledge therefore, we describe the first chemical synthesis of **2** as a single isomer, its enzymatic conversion to the potent second messenger **1**, and both chemical characterization and biological proof of the integrity of the latter dinucleotide.

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-nicotinamide mononucleotide (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**[9] and may be involved in its degradation.[10] 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.[6,11] 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*(benzyloxy)-*N*,*N*-diisopropylaminophosphane **6** (Scheme 1), to afford the respective phosphate **7** in high yield after oxidation of the intermediate phosphite with *m*-chloroperoxybenzoic acid (*m*CPBA). Imidazolium triflate[12] was used instead of tetrazole to produce a less reactive phosphytilating 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.[13]

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(benzyloxy)phosphate **7**, then treatment of the product with trisimidazolylphosphane, generated in situ from PCl_3 and imidazole, rapidly furnished **8** in quantitative yield (Scheme 1). Oxidation was best achieved by using conditions reported by Sekine and co-workers[14] in a process monitored by ^{31}P NMR spectroscopy, such that the *H*-phosphonate triethylammonium salt ($\delta(^{31}P) = 5.5$ ppm) was treated with *N*,*O*-bis(trimethylsilyl)acetamide (BSA) to afford the bis(trimethysilylphosphonate) ($\delta(^{31}P) = 116$ ppm) after approximately 1 h; the oxidant (1*R*)-(+)-(10-camphorsulfonyl)oxaziridine (CSO) was added to this to generate *bis*(trimethylsilyl)phosphate ($\delta(^{31}P) = -16$ ppm) in about 20 min, which



Scheme 1. a) Nicotinamide, CH_3CN, then TMSOTf, RT; 1.5 h, then MeOH, RT; 1 h; b) $POCl_3$, $PO(OMe)_3$, 0 °C, 4 h; c) **6**, CH_2Cl_2, RT; 3 h, then *m*CPBA, −78 °C, 0.5 h, 98%; d) TFA/H_2O/THF 1:1:4, 0 °C, 3 h, 98%; e) PCl_3, imidazole, Et_3N, THF, 0 °C, 15 mins, then 1 M TEAB aq. pH 7, RT, 15 mins, quant.; f) BSA, $CHCl_3$, RT, 1 h, then CSO, RT, 0.5 h, then MeOH/D_2O, RT, 15 mins, 72%; g) β-NMN, carbonyl diimidazole, Et_3N, DMF, RT, 3 h, then 9, DMF, RT, 16 h; h) 1 M TBAF/THF, AcOH, 0 °C, 1.5 h, 22% i) 10% Pd/C, cyclohexadiene, MeOH/H_2O, RT, 2 h, 77%; j) Nicotinic acid, *Aplysia* ADP-ribosyl cyclase, 1 M NaOAc aq. pH 4, RT, 5 h, 63%.

slowly hydrolyzed to give the phosphate **9** ($\delta(^{31}P) = 1$ ppm) upon addition of D_2O.

Successful repetition of the approach to NAD reported by Lee and co-workers[6b] encouraged initial exploration of morpholidate activation of the precursor **8**, but attempted conversions were not productive. Production and reaction of the β-NMN imidazolide could be monitored by using ^{31}P NMR spectroscopy ($\delta(^{31}P) = -10.6$ ppm).

Contrary to other reports,[6] 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 β-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 [enzyme commission number E.C.3.2.2.5] prepared from *Aplysia* ovotestis[15] to provide NAADP **1** in 63% yield.

The ability of **1** to induce release of Ca^{2+} ions was tested by using 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.[16] Synthetic NAADP, carefully quantified by using total phosphate assay prior to evaluation,[17] 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).

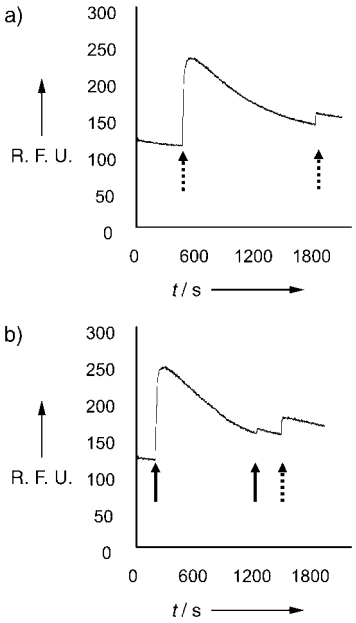


Figure 2. Ca^{2+}-ion release from homogenate of sea-urchin eggs by 100 nM authentic NAADP (dotted arrows) or 100 nM synthetic NAADP, **1** (solid arrows): Samples diluted to 2.5% in GluIM in the presence of regenerating system and kept at 17°C with agitation for 3 h to facilitate Ca^{2+}-ion uptake 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 arbritrary units; $n = > 3$ for each point. R. F. U. = relative fluorescence units.

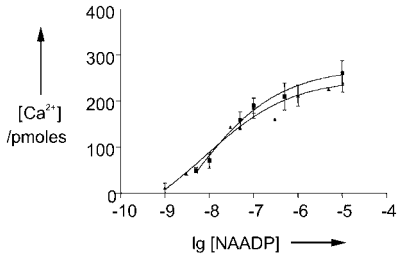


Figure 3. Inhibition of NAADP-induced Ca^{2+}-ion release by authentic NAADP (squares) or synthetic NAADP **1** (triangles) in the homogenate of sea-urchin eggs: Samples diluted to 2.5% in GluIM in the presence of a regenerating system and kept at 17°C with agitation for 3 h to facilitate Ca^{2+}-ion uptake into stores. Ca^{2+}-ion release determined by an increase of Fluo-3 fluorescence at 526 nm; data expressed as pmoles of Ca^{2+}-ions released; $n = > 3$ for each point.

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

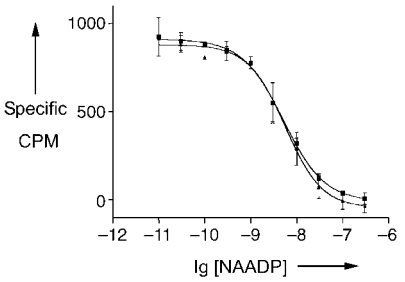


Figure 4. Competitive displacement of [^{32}P]NAADP (0.2 nM) with authentic NAADP (squares) or synthetic NAADP **1** (triangles) from sea-urchin homogenate: Samples diluted in GluIM, then 0.2 nM [^{32}P]NAADP in the presence of increasing NAADP added and incubated at room temperature for 20 mins. Samples filtered through Whatman GF/B filters to separate bound and free [^{32}P]NAADP ligand. Nonspecific binding is defined by incubation of the homogenate in the presence of 10 μM NAADP; $n = 3 \pm$ SEM; data expressed as specific cpm.

messenger pathway. Enzymatic conversion into **1** is confirmed both by identical behavior when evaluated for Ca^{2+}-release properties against sea-urchin-egg homogenate and spectroscopic characterization. Expansion of the route to include suitably protected beta-nicotinic acid mononucleotide (β-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 H_2O:MeOH (3:1, v/v, 2 mL) were stirred at room temperature under an argon atmosphere for 3 h, after which the palladium was filtered and the resulting filtrate subject to ion-exchange chromatography (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%): ^{1}H NMR (400 MHz, D_2O): $\delta = 9.29$ (s, 1H; H_N2), 9.11 (d, $J = 6.3$ Hz, 1H; H_N6), 8.78 (d, $J = 8.2$ Hz, 1H; H_N4), 8.41 (s, 1H; H_A8), 8.23 (s, 1H; H_A2), 8.13 (m, 1H; H_N5), 6.11 (d, $J = 5.9$ Hz, 1H; H_A1'), 6.01 (d, $J = 5.1$ Hz, 1H; H_N1'), 4.96 (m, 1H; H_A2'), 4.49 (m, 1H; H_A3'), 4.44 (br s, 1H; H_N4'), 4.40 (m, 1H; H_N2'), 4.31 (m, 1H; H_N3'), 4.27 (m, 1H; H_A4'), 4.26 (m, 1H; $H_A5'_a$), 4.12 ppm (m, 3H; $H_A5'_b$, H_N5'); ^{31}P NMR (162 MHz, D_2O): $\delta = -0.73$ and -11.42 to -11.84 ppm (br m); MS [FAB+]: *m/z* (%): 744.0 (50) [M^+−H]; found 744.0842 [M^+−H] $C_{21}H_{29}N_7O_{17}P_3$ requires 744.0833.

1: NADP **2** (4 mg 5 mM) and 100 mM nicotinic acid (12 mg) in a 100 mM aqueous AcOH/NaOH (pH 4, 1 mL) were incubated with 5 μL of ADP-ribosyl cyclase at room temperature. After 5 h, HPLC analysis (AG MP-1, aqueous TFA) showed complete consumption of NADP and formation of NAADP ($R_T = 16.8$ mins). The crude mixture was purified on an ion-exchange resin (AG MP-1) by using an aqueous TFA gradient (150 mM), the product eluted at 15% TFA. Combined fractions were evaporated under vacuum at room temperature and lyophilized overnight to afford **1** as a white powder (2.5 mg, 63%): ^{1}H NMR (400 MHz, D_2O): $\delta = 9.32$ (s, 1H; H_N2), 9.17 (d, $J = 5.9$ Hz, 1H; H_N6), 8.87 (d, $J = 8.1$ Hz, 1H; H_N4), 8.45 (s, 1H; H_A8), 8.28 (s, 1H; H_A2), 8.14 (m, 1H; H_N5), 6.14 (d, $J = 5.5$ Hz, 1H; H_A1'), 6.03 (d, $J = 5.1$ Hz, 1H; H_N1'), 4.97 (m, 1H; H_A2'), 4.50 (m, 1H; H_A3'), 4.44 (br, 1H; H_N4'), 4.41 (m, 1H; H_N2'), 4.30 (m, 1H; H_N3'), 4.27–4.22 (m, 2H; H_A4' and $H_A5'_a$), 4.12–4.10 ppm (m, 3H; $H_A5'_b$, H_N5'); ^{13}C NMR (100 MHz, D_2O): $\delta = 165.4$ (CO_2H), 149.8 (C_A4), 148.3 (C_A6), 147.0 (CH_A2), 144.6 (CH_N6), 142.7 (CH_N4), 142.5(CH_A8) 141.8 (CH_N2), 133.8 (C_N3), 128.6 (CH_N5), 118.4 (C_A5), 99.7 (CH_N1'), 86.8 (CH_A4'), 83.9 (CH_A1'), 77.4 (CH_N2'), 76.8 (CH_A2'), 73.8 (CH_N4'), 70.3 (CH_N3'), 69.9 (CH_A3'), 65.1 and 64.8 ppm ($2 \times CH_2$); ^{31}P NMR (162 MHz, D_2O): $\delta = 1.47$ and -9.40 to -9.80 ppm (br m); MS [FAB+] 744.8 [M^+−H]; found 745.0658 [M^+−H $C_{21}H_{29}N_7O_{17}P_3$ requires 745.0673.

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