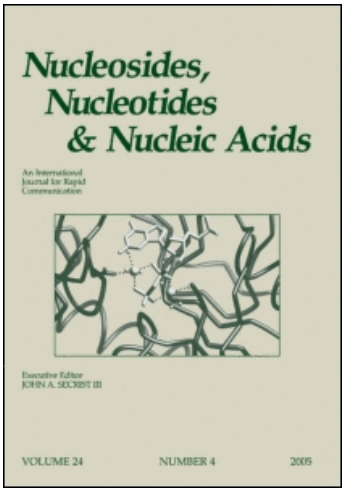

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CHEMICAL SYNTHESIS OF THE NOVEL Ca^{2+} MESSENGER NAADP

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▫ *The first total chemical synthesis of nicotinamide adenine dinucleotide phosphate (β-NADP, 2) as a single isomer was achieved. This was subsequently converted into the important second messenger nicotinic acid adenine dinucleotide phosphate (β-NAADP) 1 and the identity of this material confirmed by biological evaluation. This flexible synthetic route offers new opportunities for the generation of NAADP 1 analogues that cannot be generated directly from NADP 2 or mainly enzymatic methods.*

RESULTS AND DISCUSSION

Nicotinic acid adenine dinucleotide phosphate (NAADP, Figure 1) **1**, originally identified as a minor contaminant of commercial preparations of NADP **2**, potently induces calcium release in mammalian tissue, such as heart, T-cells, and both pancreatic acinar and beta-cells at concentrations of 10–100 nM.[1,2] It is interesting that NAADP **1** causes self-inactivation of this calcium release pathway in certain cells. Glucose stimulation of pancreatic beta-cells leads to increased endogenous levels of NAADP **1**, suggesting its role as a second messenger[3] but whether NAADP **1** addresses a discrete calcium pool to those of other messengers, such as cyclic adenine dinucleotide phosphate ribose (cADPR), remains contentious. The biological machinery that governs the intracellular chemistry of NAADP **1** is poorly characterized. Meanwhile, the high potency of **1** means that potential contamination of analogues prepared by direct modification of NADP **2**

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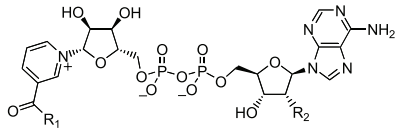


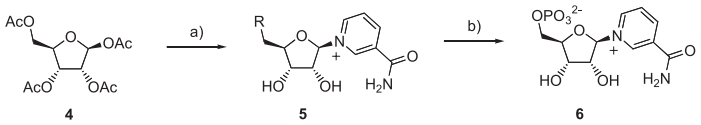
1 NAADP; $R_1 = O^-$; $R_2 = OPO_3^{2-}$
2 NADP; $R_1 = NH_2$; $R_2 = OPO_3^{2-}$
3 NAD; $R_1 = NH_2$; $R_2 = OH$

FIGURE 1

can never be ruled out, further, important analogues such as C-glycosides cannot be prepared in this way. We therefore sought to develop a flexible synthetic route toward a range of chemical probes to this end. While NAD **3** has been prepared on a kilogram scale,[4] the synthesis and isolation of NADP **2**, or NAADP **1** as a single isomer has not been published. We report the first chemical synthesis of NADP **2**, its conversion to NAADP **1**, and confirmation of its identity by biological evaluation as an important milestone toward the preparation of chemical probes.

A convergent route culminating in penultimate pyrophosphate bond formation was expected to deliver sufficient flexibility for later production of analogues. β-Nicotinamide mononucleotide (β-NMN, **6**) was therefore generated with trimethylsilyl trifluoromethanesulfonate activation of 1,2,3,5-tetra-*O*-acetyl-D-ribofuranose **4** facilitating installation of nicotinamide. Subsequent in situ methanolysis provided nicotinamide riboside, **5**, in good yield, which was converted to β-NMN, **6**, upon reaction with $POCl_3$ in trimethyl phosphate[4,5] (Scheme 1).

The 2′-phosphate adenosyl ribose motif is important for the activity of NAADP **1** but makes the synthesis somewhat more complex than that of NAD analogues because the phosphate must be installed regioselectively and maintained without migration. The 1,1,3,3,-tetraisopropyl disiloxane protected adenosine **7** allowed simultaneous protection of 3′-and 5′-hydroxyls and unambiguous, robust installation of the 2′-phosphate. 1,3-Dichloro-1,1,3,3-tetraisopropyl disiloxane was reacted with adenosine in pyridine providing good yields of the protected precursor, **7**. Imidazolium triflate catalysed reaction with phosphoramidites, such as **8**, occurred exclusively at the hydroxyl group in high yields (98% for *bis*(benzyloxy)phosphate **10**) after one pot oxidation using *m*-chloroperoxybenzoic acid (*m*CPBA). Imidazolium triflate is less reactive than more commonly used 1*H*-tetrazole such that protection of the purinyl amine is not required. Exclusive protodesilylation at the 5′-silyl ether only unveiled the 5′-alcohol **11**, using

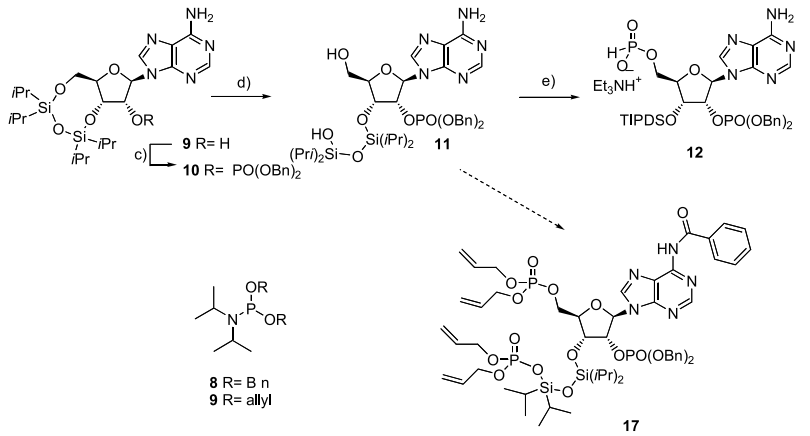


SCHEME 1 Reagents & conditions: a) Nicotinamide, CH_3CN, then TMSOTf, RT, 1.5 h; then MeOH, RT; 1 h; b) $POCl_3$, $PO(OMe)_3$, 0°C, 4 h.

carefully controlled acidic conditions.[6] Phosphoramidite reagents were again explored for installation of orthogonally protected 5′-phosphates. Reaction times were generally much slower, presumably a result of the more crowded steric environment. That a number of reagents unexpectedly proved to be incompatible with this route was a source of significant frustration. *Bis*(prop-2-enyloxy)-*N,N*-diisopropylaminophopshane **9,** reacted only sluggishly when imidazolium triflate was used as catalyst. 1*H*-tetrazole appeared to provide smooth reaction after appropriate precautions were taken to protect the purinyl amine, but led to material of higher mass than expected. Examination of the ^{1}H NMR spectrum showed that the allylic protons integrated to a total of four, rather than the expected two, possibly due to the formation of an unexpected silanol phosphate **17**. This functional group proved to be relatively robust and we were not able to convert this product into the desired precursor (Scheme 2).

Instead, *tris*-imidazolylphosphine was generated in situ from PCl_3 and imidazole and reacted with the 5′-hydroxyl, rapidly furnishing the *H*-phosphonate **12**, providing a convenient alternative route that proceeded in high yield. Subsequent oxidation was carried out using conditions originally reported by Sekine and coworkers[7] with monitoring by ^{31}P NMR. The *H*-phosphonate triethylammonium salt ($\delta(^{31}P)$ = 5.5 ppm) was converted to the *bis*(trimethylsilylphosphonate) ($\delta(^{31}P)$ = 116 ppm) after treatment with *N,O-bis*(trimethylsilyl)acetamide for approximately 1 h. Addition of (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine (CSO) resulted in oxidation to the *bis*(trimethylsilyl)phosphate ($\delta(^{31}P)$ = −16 ppm) after approximately 20 min, which was then slowly hydrolized to the phosphate ($\delta(^{31}P)$ = 1 ppm) upon addition of D_2O. Using this route we were able to obtain the phosphate precursor **13**, from adenosine in 72% yield (69% from **9**) (Figure 2).

Our initial attempts to generate the phosphomorpholidate from protected adenosine phosphate were low yielding and did not encourage further exploration



SCHEME 2 Reagents & conditions: c) **6**, CH_2Cl_2, RT; 3 h, then mCPBA, −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.

of this route. Instead, contrary to other reports,[4] we found that β-NMN imidazolide (**14**, $\delta(^{31}P) = -10.6$ ppm) reacted with good conversion when monitored by ^{31}P NMR spectroscopy. Unfortunately, the pyrophosphate product **15** is amphiphilic and purification by HPLC or ion exchange chromatography was sufficiently difficult to force further manipulation of the crude. Treatment of intermediate **15** with an acetic acid buffered solution of 1M TBAF in THF effected simultaneous cleavage of the silanol ether and one of the phosphate benzyl protecting groups, allowing ion exchange purification of the resulting monobenzyl NADP **16**. This was the only compound detectable using this purification method, suggesting that no migration of the 2′-phosphate had occurred. The best yield for this reaction was 22% over these two steps, and although this yield is modest, it is comparable with many literature reports for pyrophosphate bond formation (Scheme 3).

Ultimately, transfer hydrogenation effected clean removal of the remaining benzyl-protecting group, providing clean NADP **2** in a reasonable 77% yield after purification using ion exchange chromatography. In seeking a route to NAADP **1**, we sought to confirm the identity of the synthetic NADP, by subjecting it to base exchange in the presence of nicotinic acid and crude *Aplysia* ADP ribosyl cyclase [E.C.3.2.2.5] to provide NAADP **1**, in 63% isolated yield.

Vesicles derived from intracellular stores remain intact and sequester calcium when supplemented with and ATP-regenerating system. Using a calcium reported dye fluo-3 and cuvette-based fluorimetry, concentration-dependent calcium release can be observed when the homogenate is challenged with appropriate second messengers. After careful quantification by total phosphate assay, the identity of the synthetic NAADP **1** was checked by comparing its ability to induce Ca^{2+} release from aliquots of cell-free sea urchin egg homogenate with that of authentic material.

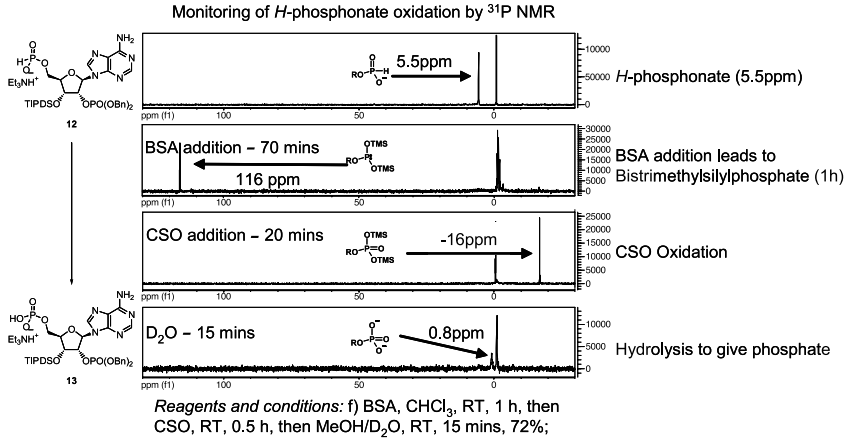
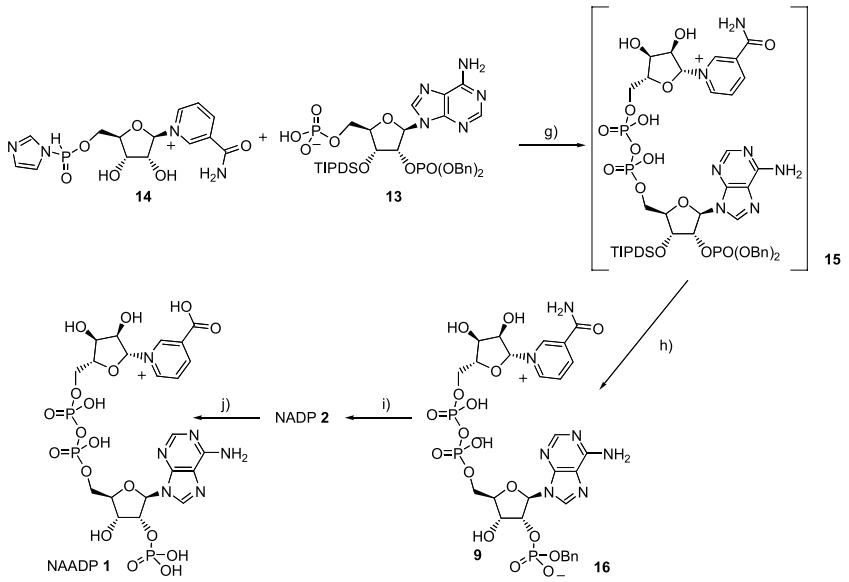


FIGURE 2



SCHEME 3 Reagents & conditions: g) DMF, RT, 16 h; h) 1M 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, 1M NaOAc aq. pH 4, RT, 5 h, 63%.

The synthetic material **1** behaved in an identical manner to authentic NAADP, inducing calcium release at 100 nM concentrations and potently causing time-dependent inactivation of calcium release at subthreshold concentrations.

In summary, we have completed the first synthesis of NADP **2** and converted this material into the second messenger NAADP.[1] This route provides robust and high yielding synthesis of adenosine analogues that furnishes and maintains the important 2′-phosphate functional group, without apparent migration. Synthesis of the β-NMN, **6** portion of this molecule is more challenging, however, and we are currently engaged in developing efficient synthesis of analogous nicotinic acid structures toward total chemical synthesis of NADP. The latter pyrophosphate bond formation and penultimate protecting group removal provides sufficient material for biological evaluation.

Much remains to be learned about the proteins that govern the intracellular chemistry of this interesting second messenger and we recognize a need for NAADP analogues that can be used as chemical probes to interrogate its chemical biology. This first total synthesis of NADP represents a significant milestone toward this wider objective.

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