ChemComm

# A chemical synthesis of nicotinamide adenine dinucleotide ($NAD^+$)

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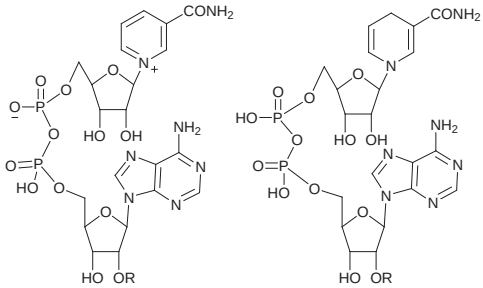
***Received (in Cambridge, UK) 18th February 1999, Accepted 10th March 1999***

**A practical synthesis of nicotinamide mononucleotide (β-NMN) and a high yield coupling with AMP-morpholidate that also provides $NAD^+$ in an efficient manner are reported.**

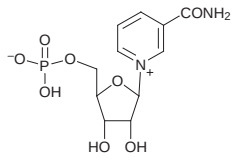
The nicotinamide cofactors, $NAD^+$, $NADP^+$, NADH and NADPH, are useful in a variety of enzyme-catalyzed oxidation and reduction reactions in organic synthesis. In the course of bioconversion research in our laboratories, there was need for a reliable, practical synthesis of these cofactors. $NAD^+$ **1**, also known as Coenzyme 1, is currently manufactured by a yeast based fermentation process.[1] An early chemical synthesis of $NAD^+$ was reported by Todd *et al*.[2] In their approach, dicyclohexylcarbodiimide (DCC) was used for the coupling of adenosine monophosphate (AMP) and β-nicotinamide mononucleotide (β-NMN, **5**). In a semi-synthetic approach to $NAD^+$



**1** $NAD^+$, R = H
**2** $NADP^+$, R = $PO(OH)_2$



**3** NADH, R = H
**4** NADPH, R = $PO(OH)_2$

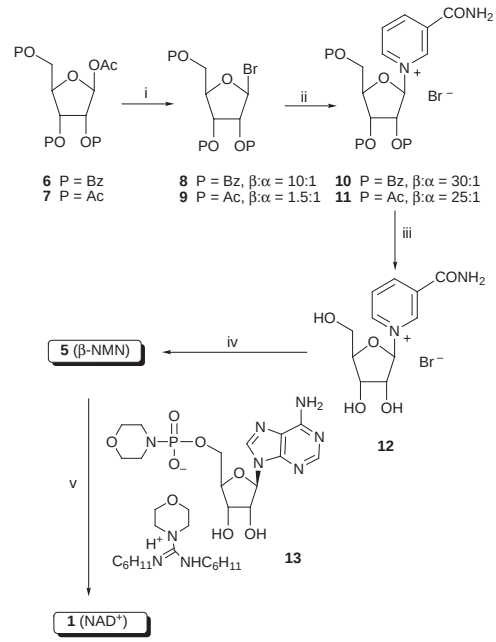


**5** β-NMN

by Whitesides *et al*., β-NMN was reacted with adenosine triphosphate (ATP) using NAD pyrophosphorylase (NADPP) immobilized in a polyacrylamide gel.[3b] Since this condensation required a stoichiometric amount of ATP, a costly reagent, a method to generate ATP from AMP was developed. This required the use of several enzymes and associated reagents, such as acetyl phosphate, acetyl kinase, adenyl kinase, and NAD-pyrophosphorylase.[3b] We envisioned that AMP could be activated as a phosphoroamidate and would react with β-NMN or *vice versa*. For this purpose we first examined the existing methods for preparing β-NMN. The β-NMN synthesis reported by Todd *et al.*,[2,3] was improved by Mikhailopulo and co-workers in terms of β-selectivity in the *N*-glycosylation step using $SO_2$ as a solvent.[4]

We found, however, that the deprotection of tribenzoate **10** under the literature conditions ($NH_3$, MeOH) was not easily repeated on a large scale (Scheme 1). We observed substantial epimerization giving a *ca.* 2 : 1 β : α mixture of anomeric nucleosides **12**. After considerable effort to improve the deprotection of the tribenzoate, we decided to define a more suitable protecting group with the following requirements: (i) readily removable in the presence of the reactive anomeric pyridinium group; (ii) stable to the bromination conditions; and (iii) able to provide neighboring group participation during the glycosylation step. Nicotinamide triacetate **11** was identified as a potential candidate and was prepared as follows. Bromination of commercial tetra-*O*-acetyl-β-D-ribofuranose **7** with HBr in $CH_2Cl_2$ gave a 1.5 : 1 β : α mixture of anomeric bromides **9**. Condensation of **9** with nicotinamide in $SO_2$ at −10 °C followed by a crystallization from 5 : 1 acetone–Bu$^t$OMe afforded the desired β-nicotinamide mononucleoside **11** in 90% overall yield from **7**. We examined other solvents for the glycosylation step. Although a 3.3 : 1 (β : α) anomeric mixture was obtained in MeCN, the pure β-anomer was selectively crystallized in 65% yield from the reaction mixture (−15 °C). Although lower yielding, this procedure provides an alternative to $SO_2$.

Clean deprotection of tri-*O*-acetate **11** was elusive. A substantial amount of nucleoside cleavage at the glycosidic linkage was observed on treatment with methanolic ammonia. After several attempts, it was learned that the extent of cleavage was largely dependent on the initial concentration and the total amount of ammonia in the reaction mixture. Under the optimized conditions (3 equiv. of $NH_3$, 1 M in MeOH, −3 to −5 °C for 20 h), deacetylation followed by a crystallization (1 : 5 MeOH–acetone), gave a single β-nicotinamide nucleoside



**6** P = Bz
**7** P = Ac
**8** P = Bz, β:α = 10:1
**9** P = Ac, β:α = 1.5:1
**10** P = Bz, β:α = 30:1
**11** P = Ac, β:α = 25:1
**12**
**13**

**Scheme 1** *Reagents and conditions*: i, HBr (g), $CH_2Cl_2$; ii, nicotinamide, $SO_2$, −10 °C, 90% from **7**; iii, $NH_3$, MeOH, −5 °C, 20 h, 80%; iv, $POCl_3$ (4 equiv.), $PO(OMe)_3$, −5 to 0 °C, 7 h, 80%; v, $MnCl_2$–$MgSO_4$, $HCONH_2$, room temp., 16 h, 78% (58% isolated yield, > 99% purity).

bromide salt **12** as a crystalline solid in 80% isolated yield. It is noteworthy that, contrary to the previous report,[4] we found both **11** and **12** to be air-stable and non-hygroscopic solids.

The phosphorylation of the nicotinamide triol **12** has been reported in the literature using 1 equiv. of $POCl_3$ in $PO(OMe)_3$ with variable yield (35–64%).[4,5] Accordingly, we reexamined the reaction conditions and observed that reaction was very slow below −5 °C, while at > 5 °C a chlorination reaction was the favored pathway.[6,7] Thus the triol **12** was treated with 4.0 equiv. of $POCl_3$ in $PO(OMe)_3$ between −5 and 0 °C for 7 h to give the desired phosphorylated product in 90–92% yield (Scheme 1). Upon neutralization of the reaction mixture, the crude β-NMN **5** was separated from the reaction mixture by addition of MeCN–$Et_2O$ (1 : 3). This crude product was further purified using resin chromatography to give the β-NMN in > 97% purity in 80% isolated yield.[8,9] Use of excess of $POCl_3$ (4 equiv.) at controlled temperature proved far superior to the more conventional stoichiometric amount of the reagent.

With β-NMN in hand, our attention turned to development of a practical method for pyrophosphate bond formation *en route* to $NAD^+$.[10] Conventional ways to make the pyrophosphate bonds include carbodiimide coupling,[2,11] the Michelson procedure,[12] and the Khorana–Moffatt procedure.[13] This last method, involving the coupling of a suitable salt of a sugar phosphate and a nucleotide phosphoromorpholidate, has been widely used for the synthesis of various sugar nucleotides.[13] However, the reaction between β-NMN and AMP-morpholidate[13*b*] (**13**) was attempted and gave $NAD^+$ in < 5% yield.[14] Our initial attempts using the AMP-imidazolidate,[15] triazolidate, or tetrazolidate, or to react AMP with β-NMN-imidazolidate[16] under the modified Khorana–Moffat conditions gave only low conversion to $NAD^+$ (formamide, 16 h to 6 days). We reasoned that this low reactivity of AMP-amidates might be due to the fact that β-NMN is an inner salt and is thus less nucleophilic. Under these conditions, a second dissociation of the phosphoric acid in the β-NMN, p$K_a$ ~ 7, is probably not occurring to any significant extent. Accordingly, we attempted to activate the reaction using Lewis acid additives. The coupling between β-NMN and AMP-imidazolidate in the presence of $MnCl_2$–$4H_2O$ in formamide gave the desired $NAD^+$ in 25% yield after 16 h.[16] Further fine-tuning of the reaction conditions improved conversion up to 78% (HPLC assay yield). Typically, the reaction was carried out using 1.0 equiv. of AMP-morpholidate (**13**) with 1.1 equiv. of β-NMN (**5**) in 0.2 M solution of $MnCl_2$ (1.5 equiv.)[17] and $MgSO_4$ (2 equiv.) in formamide at room temperature for 16 h. Upon completion, the crude $NAD^+$ was precipitated from the reaction mixture with MeCN, and further purified by resin chromatography (Sephadex QAE A-25, elution with 0.25 M aq. $(NH_4)HCO_3$ solution; then Amberite XAD-16 with water–MeOH gradient at 5 °C)[18] followed by freeze-drying to give the $NAD^+$ as its ammonium salt with > 99% purity in 58% isolated yield.

The practical synthesis of $NAD^+$ outlined above has a number of noteworthy steps. The mild deacetylization procedure allowed isolation of the crystalline air stable nicotinamide nucleoside. Phosphorylation conditions were developed to provide β-NMN in 80% isolated yield. A new catalyzed pyrophosphate formation between β-NMN and AMP-morpholidate using a combination of $MnCl_2$ and $MgSO_4$ gave $NAD^+$ in 78% yield. An improved resin isolation process provided $NAD^+$ in 58% isolated yield.

We are grateful to Dr R. Czaja, Mr J. Bergan and Mr Bob Reamer for technical assistance.

## Notes and references

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3 While β-NMN is commercially available, its high price ($508 per gram, Sigma 1997 catalog) and a lack of supply made large scale preparation of $NAD^+$ difficult. For other preparations of β-NMN, see D. R. Walt, V. M. Rios-Mercadillo, J. Auge and G. M. Whitesides, *J. Am. Chem. Soc*., 1980, **102**, 7806; D. R. Walt, M. A. Findeis, V. M. Rois-Mercadillo, J. Auge and G. M. Whitesides, *J. Am. Chem. Soc*., 1984, **106**, 234; G. M. Whitesides and D. R. Walt, US Pat. 4,411,995-A, 1983; R. Jeck, P. Heik and C. Woenckhaus, *FEBS Lett*., 1974, **42**, 161.

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6 In considering an efficient method for the synthesis of the pyrophosphate, we attempted to prepare the intermediate dichlorophosphate (phosphorodichloridate). Dichloridate was then treated *in situ* with AMP monosodium salt and triazole. However, no pyrophosphate bond formation was observed by $^{31}P$ NMR spectroscopy.

7 In an attempt to eliminate the bis-adduct formation, the reaction was also carried out using a 1 : 1 mixture of $POCl_3$–$H_2O$ [*in situ* generation of $(HO)POCl_2$]; see M. Yoshikawa, T. Kato and T. Takenishi, *Bull. Chem. Soc. Jpn.*, 1969, 3505.

8 Chromatography on Dowex resin (two successive columns, 1X2 formate, 50WX8 $H^+$ form) followed by subsequent lyophilization of the aqueous fractions provided β-NMN in 80% yield, 97% purity (120 g scale). The two resin column isolation process proved to be operationally simple and the Dowex 50WX8 resin ($H^+$ form) served the dual purpose of purification and, more importantly, pH adjustment to provide the desired inner salt form of β-NMN.

9 The crude β-NMN was initially purified by a resin-free isolation [*i.e.* precipitation followed by treatment with activated carbon (Ecorsob-C, P-502-H)]. Although the process is simple, the yield for the next coupling reaction with AMP-morpholidate under the optimized condition was only moderate (it still required pH adjustment using $H^+$ cation resin). Alternatively, a direct resin chromatography using Dowex (50W X 8-100) resin column was developed (97% purity, eluted with 5% formic acid in water). However, this procedure required high resin loads with the product eluting in a large volume of water.

10 For examples of general pyrophosphate bond formation, see: A. F. Cook, M. J. Holman and A. L. Nussbaum, *J. Am. Chem. Soc*., 1969, **91**, 1522; K. Furusawa, M. Sekine and T. Hata, *J. Chem. Soc., Perkin Trans. 1,* 1976, 1711; F. Cramer and H. Schaller, *Chem. Ber*., 1961, **94**, 1634; V. J. Davisson, A. B. Woodside, T. R. Neal, K. E. Stremler, M. Muehlbacher and C. D. Poulter, *J. Org. Chem*., 1986, **51**, 4768; J. R. Falck, K. K. Reddy, J. Ye, M. Saady, C. Mioskowski, S. B. Shears, Z. Tan and S. Safrany, *J. Am. Chem. Soc*., 1995, **117**, 12172; V. Wittmann and C.-H. Wong, *J. Org. Chem*., 1997, **62**, 2144.

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17 For a successful high yielding coupling reaction, reagents and the solvents were dried. A $MnCl_2$ solution was prepared by dissolution of the commercial tetrahydrate in formamide to give a 0.2 M stock solution. The solution was dried over 4 Å molecular sieves for several days prior to use. Typically the solution had 0.5 M $H_2O$ content and was used for the reaction successfully. Use of commercial anhydrous $MnCl_2$ proved inferior.

18 Purification of $NAD^+$ using resin chromatography at room temperature resulted in partial decomposition of purified $NAD^+$ (5% purity loss). Therefore, the chromatography was carried out at 5 °C (110 g scale, > 99% purity).

*Communication 8/09930H*