Syntheses of Nicotinamide Riboside and Derivatives: Effective Agents for Increasing Nicotinamide Adenine Dinucleotide Concentrations in Mammalian Cells

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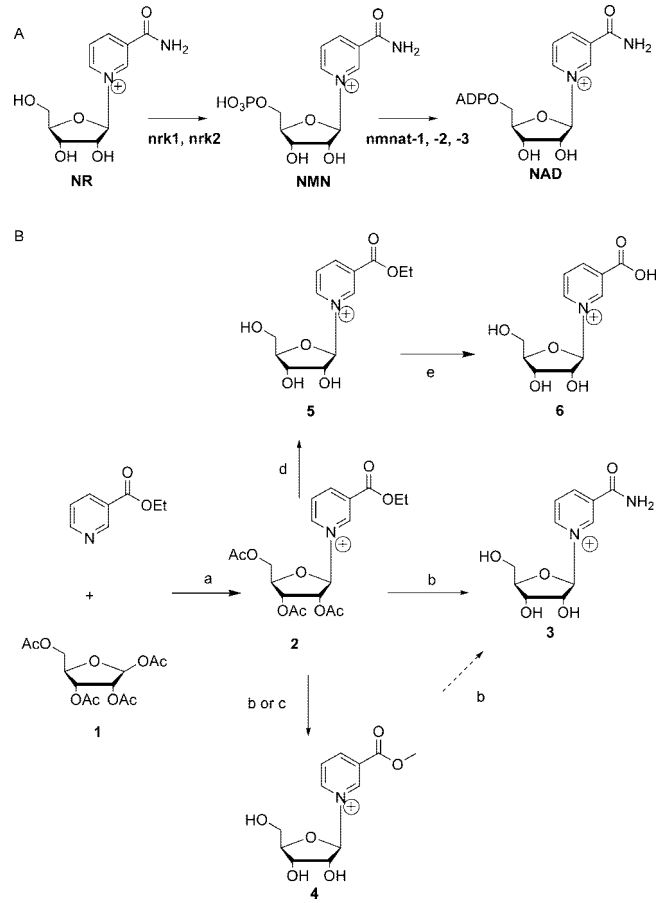
Abstract: A new two-step methodology achieves stereoselective synthesis of β-nicotinamide riboside and a series of related amide, ester, and acid nucleosides. Compounds were prepared through a triacetylated-nicotinate ester nucleoside, via coupling of either ethylnicotinate or phenylnicotinate with 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose. Nicotinamide riboside, nicotinic acid riboside, *O*-ethylnicotinate riboside, *O*-methylnicotinate riboside, and several *N*-alkyl derivatives increased NAD^+ concentrations from 1.2–2.7-fold in several mammalian cell lines. These findings establish bioavailability and potent effects of these nucleosides in stimulating the increase of NAD^+ concentrations in mammalian cells.

A resurgent interest in NAD^+[a]metabolism has resulted in a number of recent reviews,[1–3] and speculation that this long-studied metabolism could be fertile ground for discovery of new therapies for the diseases of aging such as diabetes and neurodegenerative disorders.[1–3] A major reason for this increased interest is the establishment of a connection between NAD^+ metabolism and the beneficial effects of calorie restriction (CR).[4] CR increases lifespan in a number of model organisms and has been shown to require NAD^+-dependent sirtuins in yeast, flies, and worms.[4] Evidence indicates that sirtuins and NAD^+ metabolism in humans (7 homologues SIRT1−SIRT7) also respond to CR[4] and provide beneficial effects such as increased stress resistance,[5,6] mitochondrial biogenesis,[7,8] and regulation of endocrine pathways and basic metabolism.[7–9] Sirtuins also have potent effects in regulating cellular proliferation and differentiation.[4] A seminal idea has emerged that increased NAD^+ concentrations may potentiate mammalian sirtuin functions.[4] For example, increased intracellular NAD^+ concentration has been shown to activate the sirtuin SIRT1 in brain,[10] and has been proposed to help explain the protective effects of CR in a mouse model of Alzheimers.[10] In addition, recent studies have determined protective effects of several NAD^+ precursors like nicotinamide, nicotinic acid mononucleotide, nicotinamide mononucleotide (NMN), β-nicotinamide riboside (NR), and NAD^+ in neurons under conditions of genotoxicity or trauma.[11–13] The pro-survival benefits of sirtuins and the beneficial effects seen for stimulation of NAD^+ metabolism in cells have raised the question if pharmacological agents that raise NAD^+ concentrations might provide therapeutic benefits.[2,3]

We sought to evaluate NR and derivatives as a means to increase NAD^+ concentrations in mammalian cells. NR is metabolized to NAD^+ in bacteria,[14] yeast,[15] and mammals.[16] NR in human cells is thought to be metabolized by two nicotinamide riboside kinases (Nrk1 and Nrk2),[16] which phosphorylate NR on the 5′-OH of the ribose ring using ATP as a phosphoryl donor to form NMN (Scheme 1A).[16] NMN is then adenylated by NMN adenylytransferases (Nmnat-1, Nmnat-2, or Nmnat-3) to form NAD^+ (Scheme 1A).[2] Tiazofurin and benzamide riboside, which are stable analogs of NR and used clinically as anticancer agents, are proposed to be metabolized similarly to form cytotoxic NAD^+ analogs.[2,16,17]

The metabolism of NR in mammalian cells was first studied by Kornberg[18] who reported in 1951 his investigations to determine if NR could be converted to NMN.[18] In examining liver extracts he identified a phosphorylysis activity that degraded NR to ribose-1-phosphate as well as an ATP-dependent activity able to synthesize NMN. This work anticipated the discovery of the human Nrk enzymes by 40 years.[16,18] Since then, there have been few reports, until just recently, evaluating the biological properties of NR or *N*-nucleoside derivatives of NR in mammalian cells, in spite of the fact that a number of *C*-nucleoside analogs of NR have been synthesized and evaluated for cytotoxicity.[17] This situation could be partly

Scheme 1. (A) Direct Metabolism of Nicotinamide Riboside to NAD^+ within Mammalian Cells. (B) Synthesis of **2** and Transformations to Nicotinamide Riboside **3**, Nicotinate Ester Ribosides **4** and **5**, and Nicotinic Acid Riboside **6**[a]



[a] Reagents and conditions: (a) CH_2Cl_2, TMSOTf, reflux, 4 h; (b) NH_3/MeOH, 4 °C; dotted line represents proposed path of formation of NR at 4 °C, as discussed in text; (c) MeO^-/MeOH, −20 °C; (d) EtO^-/EtOH, −20 °C; (e) lipase or $OH^-_{(aq)}$.

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[a]Abbreviations: ATP, adenosine 5′-triphosphate; NR, nicotinamide riboside; NAD, nicotinamide adenine dinucleotide; NAMN nicotinic acid mononucleotide; NMN, nicotinamide mononucleotide; TMSOTf, trimethylsilyl trifluoromethanesulfonate.

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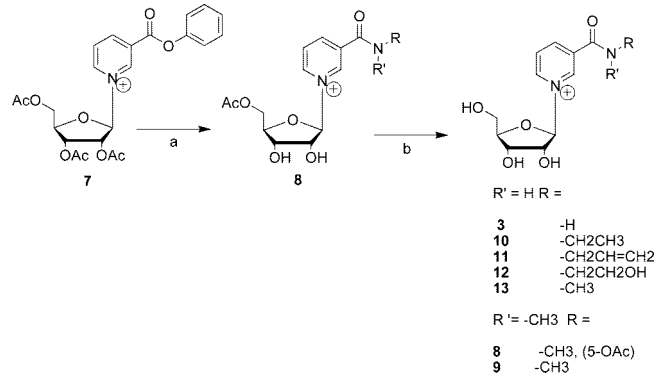
explained by limited synthetic access to NR and its derivatives. To date, there is no efficient systematic methodology available to synthesize nicotinate riboside and its derivatives, although modestly effective chemical syntheses[19–21] and an enzymatic synthesis[18] of NR have been reported. Importantly, there is no quantitative information available on the extent to which NR or nicotinate nucleoside derivatives can increase NAD^+ concentrations in mammalian cells, although a recent study indicates that NR substantially increases NAD^+ contents in yeast.[15] In this study, we report easy and flexible methods to stereoselectively synthesize NR and its acid, ester, and amide derivatives in high yield. In a significant advantage to synthetic efficiency, the methods to synthesize NR and NR derivatives utilize common synthetic intermediates. Significantly, we also report that NR and several nicotinate ester and nicotinic acid nucleosides increase cellular NAD^+ contents by amounts as high as 270% of controls, demonstrating that NR and its derivatives can potently elevate NAD^+ concentrations in mammalian cells.

Scheme 2. Synthesis of Alkyl-Substituted Nicotinamide Riboside Derivatives[a]



[a] Reagents and conditions: (a) amine/trifluoroethanol, 4 °C; (b) NH_3/MeOH, −20 °C.

β-NR was prepared with a relatively simple two-step, one-pot procedure. The synthesis was initiated by preparation of 2′,3′,5′-tri-*O*-acetyl-β-ethyl nicotinate riboside **2** (Scheme 1B) by coupling of commercially available 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (**1**) with ethyl nicotinate (1.2 equiv) in the presence of TMSOTf (1 equiv). ^{1}H NMR showed that the product was formed stereoselectively to produce only the β-isomer in a high yield (Scheme 2, >90% versus starting sugar). The clean stereochemical result suggests that the coupling proceeds via a cationic *cis*-1,2-acyloxonium-sugar intermediate, which controls addition by nucleophiles to generate predominantly β-stereochemistry.[20]

Intermediate **2** was purified for complete characterization (see Supporting Information for experimental details), but crude **2** could be used to prepare NR, **3**. Treatment of **2** with 4 N ammonia in methanol at 4 °C for 12 h provided simultaneous deprotection and conversion of the cationic nicotinate ester to the corresponding amide to furnish **3** in excellent isolated yield (Scheme 1B, 85% overall from **1**). The ease of synthesis of NR from **2** at low temperatures established that the pyridinium ester is quite reactive to substitution by amine. In contrast, ethyl nicotinate itself was unreactive under these conditions. With the pyridinium ester reactivity in mind, we considered the potential of intermediate **2** as a versatile precursor for preparation of other NR derivatives.

Progress toward the goal of using **2** as a general synthetic precursor was aided by observations made on the mechanism of amide formation. It was determined that intermediate **2** reacted in methanolic ammonia at −20 °C for 12 h (rather than 4 °C for 12 h) did not form NR as expected, but *O*-methylnicotinate-riboside, **4**, was formed instead (Scheme 1B). This result implies that **4** typically forms prior to NR at 4 °C (Scheme 1B), and that methoxide in the reaction mixture is inherently more reactive than ammonia with the pyridinium ester. Based on this finding, we investigated the concept that nicotinate ester nucleoside derivatives could be obtained from **2** by choice of an appropriate alcohol for the deprotection solvent. To avoid the possible formation of amides, we turned to sodium alkoxides to effect simultaneous deprotection and transesterification reactions.

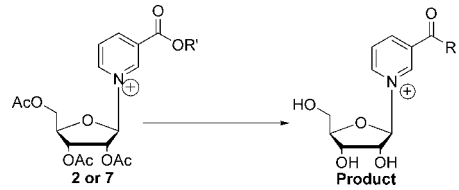
The successful application of this strategy is illustrated by two examples. Treatment of **2** with sodium methoxide in MeOH or with sodium ethoxide in ethanol at −20 °C formed *O*-methyl β-nicotinate riboside **4** (85%) and *O*-ethyl β-nicotinate riboside **5** (81%), respectively (Scheme 1B). Ester **5** could be further hydrolyzed with hydroxide or more cleanly reacted with porcine liver esterase to afford the acid derivative β-nicotinic acid riboside **6**. To our knowledge, there are no previous reports of nicotinate ester nucleosides in the literature and our report is the first synthesis and characterization of **4** and **5**. Moreover, only one chemical synthesis of **6** has previously appeared in the literature.[19]

We attempted conversion of **2** to *N*-alkyl-nicotinamide ribosides by reaction with alkylamines in methanol at 4 °C. Unfortunately, only decomposition of the nucleoside was observed and eliminated *N*-alkyl nicotinamides were major products. Thus, we prepared the more reactive phenyl ester 2′,3′,5′-tri-*O*-acetyl-β-phenyl nicotinate riboside **7** (Scheme 2) by reflux of phenyl nicotinate (see Supporting Information for synthesis) with **1** in the presence of TMS(OTf) (90% vs sugar, as determined by NMR). Ester **7** proved versatile for synthesis of several previously undescribed *N*-alkyl NR derivatives from primary and secondary amines under new conditions (Scheme 2).

Similar to the ethyl ester of **2**, the phenyl ester of **7** is more reactive with alkoxide than amine in alcoholic solvents, suggesting that transesterification to a less-reactive ester will typically precede amide synthesis. To mitigate this reaction, we turned to trifluoroethanol (TFE) as a solvent for deprotection and amide synthesis. This solvent has a pK_a of 12.5, making it 10000 times more acidic than methanol and ethanol. Reaction of **7** in 7 N dimethyl amine/TFE at 4 °C after 18 h gave the desired amide, as expected, although surprisingly, the 5′-*O*-acetyl group remained intact on the isolated nucleoside **8** (Scheme 2), as shown by ^{1}H NMR and MALDI (see Supporting Information). This selective deprotection method is currently being further explored; however, removal of the 5′-acetyl could be achieved by further addition of NH_3/MeOH (to 4 M, −20 °C for 12 h) to form the desired dimethylnicotinamide riboside **9**. Several unreported *N*-alkyl nicotinamide ribosides **10**−**13** were also prepared in good yield with these procedures (Table 1).

To examine the effect of NR on NAD^+ concentrations, we treated Neuro 2a (neuroblastoma), HEK293 (human embryonic kidney), and AB1 (mouse embryonic stem cells; used to study proliferation/differentiation pathways) with varying concentrations of NR (0–600 μM) for 48 h and then determined NAD^+ content by a known cycling assay.[22] As shown in Figure 1, NAD^+ increased with increasing NR in a dose-dependent manner in all examined cell lines, reaching 157−205% at 600 μM. All cell lines were responsive to NR, but not to the same extent. We next examined 500–1000 μM concentrations of

Table 1. Preparation of NR and its Derivatives and Effects on NAD^+ and Cell Viability



#	intermediate **2**, R′ = C_2H_5; **7**, R′ = C_6H_5	conditions	product R =	yield (%)	NAD effect[a] (fold)	cell viability[b] (%)
3	**2**	MeOH/NH_3, 4 °C	-NH_2	85	2.7 ± 0.4[c]; 1.7 ± 0.1[d]; 2.6 ± 0.3[e]	123 ± 16[c]; 111 ± 7[d]; 107 ± 12[e]
4	**2**	MeO^-/MeOH, –20 °C	-OCH_3	85	1.7 ± 0.2[c]; 2.1 ± 0.4[d]; 1.4 ± 0.3[e]	125 ± 11[c]; 94 ± 4[d]; 76 ± 10[e]
5	**2**	EtO^-/EtOH, –20 °C	-OC_2H_5	81	2.0 ± 0.7[c]; 1.4 ± 0.1[d]; 1.7 ± 0.2[e]	103 ± 3[c]; 92 ± 8[d]; 169 ± 4[e]
6	**2**	lipase or OH- from **5**	-OH	80	1.5 ± 0.5[c]; 1.9 ± 0.3[d]; 1.7 ± 0.2[e]	111 ± 14[c]; 92 ± 7[d];125 ± 21[e]
9	**7**	R_2NH/TFE, 4 °C, MeOH/NH_3, –20 °C	-$NH(CH_3)_2$	52	1.2 ± 0.3[c]; 0.63 ± 0.3[d]; 0.86 ± 0.19[e]	132 ± 25[c]; 78 ± 2[d]; 35 ± 5[e]
10	**7**	RNH_2/TFE, 4 °C, MeOH/NH_3, –20 °C	-NHC_2H_5	80	1.3 ± 0.1[c]; 1.2 ± 0.3[d]; 0.87 ± 0.10[e]	57 ± 3[c]; 48 ± 2[d]; 38 ± 4[e]
11	**7**	RNH_2/TFE, 4 °C, MeOH/NH_3, –20 °C	-$NHCH_2CH{=}CH_2$	80	2.4 ± 0.3[c]; 1.3 ± 0.2[d]; 2.6 ± 0.1[e]	57 ± 9[c]; 78 ± 1[d]; 80 ± 2[e]
12	**7**	RNH_2/TFE, 4 °C, MeOH/NH_3, –20 °C	-NHC_2H_4OH	80	2.4 ± 0.1[c]; 1.6 ± 0.3[d]; 1.6 ± 0.2[e]	101 ± 25[c]; 77 ± 9[d]; 51 ± 5[e]
13	**7**	RNH_2/TFE, 4 °C, MeOH/NH_3, –20 °C	-$NHCH_3$	80	2.1 ± 0.1[c]; 1.4 ± 0.2[d]; 0.81 ± 0.03[e]	79 ± 9[c]; 72 ± 1[d]; 76 ± 4[e]

[a] NAD^+ concentrations are relative to controls. Values for **3**, **5**, and **6** were determined by ^{18}O assay and all others were determined by cycling assay. Concentrations for each compound: **3**, 1 mM; **4**, 750 μM; **5**, 800 μM; **6**, 670 μM; **9**–**13**, 500 μM. Incubation time was 24 h. [b] Cell viability was determined by growing cells for 24 h and then treating with 750 μM compound for 48 h. Cell viability was measured by MTT assay (Supporting Information) and expressed as % vs control. Numbers above 100% suggest enhanced proliferation and values below 100% suggest retardation of cell growth and/or increased cell death. All cell experiments are in duplicate. [c] AB1 cells. [d] HEK293 cells. [e] Neuro 2a cells. RNH_2: primary amine where R is defined in product. TFE/trifluoroethanol.

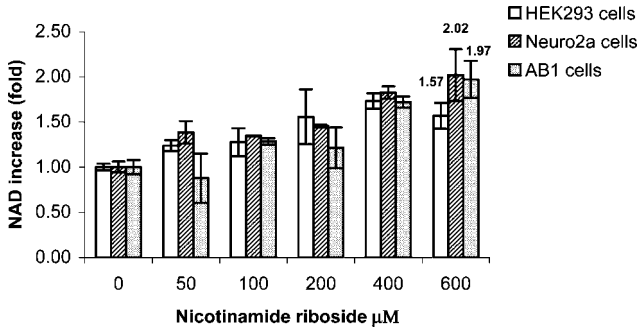


Figure 1. Effect of increasing concentrations of NR on NAD^+ in Neuro 2a, HEK293, and AB1 cells. Cells were counted by hemocytometry, and NAD^+ was assayed by a cycling assay, as described in the text. Each NR concentration for the experiment was performed in duplicate, with the standard deviation displayed by error bars. Control NAD^+ concentrations were determined to be 610 pmol/10^6 cells for AB1 cells, 320 pmol/10^6 cells for HEK293 cells, and 680 pmol/10^6 cells for Neuro 2a cells.

compounds **3**−**6** and **9**−**13** in their abilities to increase NAD^+ concentrations in cells during a 24 h period. NAD^+ contents for **3**, **5**, and **6** were measured using a MS-based, isotope-dilution technique that we have developed and used in previous studies (see Supporting Information for details).[10,23,24] Other compounds were determined by cycling assay. As shown in Table 1, compounds **3**−**6**, which includes NR, nicotinic acid riboside, and nicotinate ester nucleoside derivatives, potently increase NAD^+ contents in all cell lines, with NR the most potent (170–270%). The ester and acid derivatives increased NAD^+ contents 140–200% in all cell lines examined. Compounds **2**−**6** were relatively nontoxic to cells as measured by MTT assay (Table 1). Intermediate **2** also increased NAD^+ concentrations in all cell lines (130 ± 30%, 160 ± 10%, and 260 ± 30% for J1, HEK293, and Neuro 2a cells, respectively; no cytoxicity was observed).

Surprisingly, the *N*-substituted compounds **11**−**13** increased NAD^+ concentrations in the cell lines examined (130−240%; Table 1) with one exception (Neuro 2a with compound **13** showed a decrease to 81%; Table 1). HPLC of **11**−**13** (see Supporting Information) demonstrated them to have excellent purities and no contamination by nicotinamide, NR, or nicotinic acid. These increases in NAD^+ were also confirmed by the ^{18}O isotope method (data not shown). Compounds **9** and **10** were ineffective in stimulating NAD^+ synthesis in cells (63–120%). To assess for effects on cell growth, cells were treated with compounds for a 48 h period and then assayed by the standard MTT assay. Compounds **9** and **10** reduced growth of the neuroblastoma Neuro 2a cells to 35% and 38% of controls (Table 1). Compounds **9**−**13** showed growth inhibition in at least one cell line, and **10**, **11**, and **13** were inhibitory to all cell lines, as measured by MTT assay (Table 1). Thus, effects on cell growth and NAD^+ increase were not clearly connected for these derivatives.

In summary, we establish a new synthetic methodology for synthesis of nicotinamide riboside, nicotinic acid riboside, and several unreported *N*-alkyl nicotinamide riboside and *O*-alkyl nicotinate-riboside derivatives. This methodology provides the desired analogues using a two-step approach involving the intermediacy of a tetra-ester nicotinate riboside, which can be deprotected and reacted with alkoxides or amines to generate the desired *O*- and *N*-substituted nucleosides. The methodology typically provides overall yields of at least 80%.

Tests of NR effects on mammalian cells show that NR increases cellular NAD^+, dependent on cell line (up to 270% of control). The observed increases of NAD^+ concentrations are similar to those observed in yeast grown with NR in nicotinic acid-deficient media.[15] Several other *O*-substituted derivatives of NR increase NAD^+ in cells as well, including nicotinic acid riboside, *O*-ethyl and *O*-methylnicotinate ribosides, and triacetyl-*O*-ethylnicotinate riboside. These compounds increase NAD^+ contents 130% to 260% of control. Surprisingly, several *N*-alkyl derivatives of NR also stimulate NAD^+ synthesis. The mechanism of action for NAD^+ increase caused by these compounds is currently unknown and is under investigation in our laboratory.

Our results indicate that NR and its derivatives potently stimulate NAD^+ biosynthesis in mammalian cells. These data suggest that the biologically conserved Nrk pathway has a robust capacity to utilize NR for NAD^+ biosynthesis, although it is currently not possible to exclude other pathways for the conversion of NR or derivatives to NAD^+. For example, some

of these compounds might act via stimulation of stress signaling, which can dramatically increase cellular NAD^+,[24] or some of them might be degraded to nicotinamide or nicotinic acid,[15,18] which can be converted to NAD^+ via Nrk-independent pathways. It is concluded that NR and derivatives could provide an effective pharmacological means to increase NAD^+ concentrations in mammalian cells and tissues and may aid in the investigation of NAD^+ signaling in cells and how NAD^+ impacts survival, proliferation, differentiation, and other physiological processes.

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Supporting Information Available: Experimental procedures and compound characterization are available in a PDF file. This material is available free of charge via the Internet at http://pubs.acs.org.

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