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Emissive Synthetic Cofactors: Enzymatic Interconversions of ^{tz}A Analogues of ATP, NAD$^+$, NADH, NADP$^+$, and NADPH

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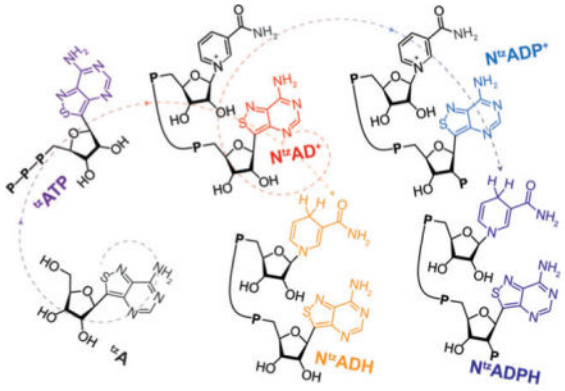
Abstract

A series of enzymatic transformations, which generate visibly emissive isofunctional cofactors based on an isothiazolo[4,3-d]pyrimidine analogue of adenosine (**^{tz}A**), was developed. Nicotinamide adenylyl transferase condenses nicotinamide mononucleotide and **^{tz}ATP** to yield **N^{tz}AD$^+$**, which can be enzymatically phosphorylated by NAD$^+$ kinase and ATP or **^{tz}ATP** to the corresponding **N^{tz}ADP$^+$**. The latter can be engaged in NADP-specific coupled enzymatic transformations involving conversion to **N^{tz}ADPH** by glucose-6-phosphate dehydrogenase and reoxidation to **N^{tz}ADP$^+$** by glutathione reductase. The **N^{tz}ADP$^+$/N^{tz}ADPH** cycle can be monitored in real time by fluorescence spectroscopy.

Relatives in the spotlight

A series of enzymatic transformations generate visibly emissive isofunctional cofactors based on an isothiazolo[4,3-*d*]pyrimidine analogue of adenosine (**^{tz}A**, see figure). These synthetic cofactors may find utility as tools for monitoring redox reactions in vitro or potentially in living systems.



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Conflict of interest

Professor Tor provides consulting services to TriLink Bio-technologies. The terms of the arrangements have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies.

Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.201711935. **^{tz}A:** isothiazolo[4,3-*d*]pyrimidine analogue of adenosine.

Keywords

cofactors; kinases; NAD^+; $NADP^+$; nucleotides

Nucleic acids and their building blocks play central roles in cellular events, including storage, retrieval, regulation, and expression of genetic information, as well as signaling and metabolic pathways. This extensive biology necessitates the development of tools for studying their recognition features as well as their utilization and alteration by endogenous and exogenous agents.[1] Emissive nucleoside analogues, when judiciously fabricated and incorporated, could serve as effective photophysical and mechanistic probes.[2] Extensive efforts have indeed been dedicated to the development and refinement of such means.[3–5]

Our focus over the past decade has been on the design, synthesis, and implementation of new emissive nucleoside analogues that can faithfully replace their natural counterparts.[3,6] A guiding principle for the successful advancement of such probes is to curtail structural and functional perturbations (features defined as isomorphicity and isofunctionality, respectively), while endowing them with useful photophysical attributes. Several families of emissive analogues have been made and employed,[6] with emissive RNA alphabets based on the thiopheno[3,4-*d*]pyrimidine (**thN**)[7] and the isothiazolo[4,3-*d*]pyrimidine-cores (**^{tz}N**)[8] being most notable.[9–11]

While the incorporation of a single modified nucleoside into an oligonucleotide could be structurally and functionally benign, as detrimental effects might be masked, the adequate performance of nucleosides and nucleotides as cofactors and secondary messengers represents a demanding test for their isomorphism and isofunctionality.[12] We have thus initiated the exploration of synthetic emissive cofactors and their metabolic interconversions by transferases, kinases, and hydrolases, with the goal of defining their biological recognition space and potential utility for investigating signaling and metabolic pathways.[13] Since the native NAD^+, and $NADP^+$ are nonemissive, developing and implementing isomorphic and isofunctional fluorescent surrogates will allow one to monitor their biochemical transformation in real time using common fluorescence spectrometers.[13b] As the synthesis of such cofactors is frequently challenging, their adoption by the community tends to lag behind. We have therefore sought to advance enzymatic pathways to their preparation, which could be employed in any laboratory using available enzymes[14] and monitored by conventional steady-state fluorescence measurements.

Here we illustrate the enzymatic synthesis of the emissive and isofunctional **$N^{tz}AD^+$** from nicotinamide mononucleotide and **^{tz}ATP**.[14] We then show that **$N^{tz}AD^+$** can be enzymatically phosphorylated by NAD^+ kinase to the corresponding **$N^{tz}ADP^+$**, which in turn can be engaged in NADP-specific coupled enzymatic transformations involving conversion to **N^{tz}ADPH** by glucose-6-phosphate dehydrogenase and reoxidation to **$N^{tz}ADP^+$** by glutathione reductase. Rewardingly, the **$N^{tz}ADP^+$/N^{tz}ADPH** cycle can be monitored in real time by emission spectroscopy.

A key precursor for the enzymatic fabrication of emissive adenosine-containing cofactors is isothiazolo-adenosine tri-phosphate (**^{tz}ATP**),[14] which was synthesized from the

corresponding nucleoside according to common protocols (Scheme 1).[15] To evaluate the biocompatibility of **^{tz}A**-based cofactors, a multienzymatic assay was used in which a mixture of nicotinamide mononucleotide (NMN) and **^{tz}ATP** is treated with nicotinamide adenylyl transferase 1 (NMNAT-1) and subsequently with *S. cerevisiae* alcohol dehydrogenase (ADH). The former enzyme catalyzes the transfer of nicotinamide mononucleotide to ATP,[16] releasing inorganic pyrophosphate, while the latter oxidizes ethanol to acetaldehyde in the presence of NAD^+ as a cofactor (Figure 1a). Thus, to enzymatically access **$N^{tz}AD^+$**, **^{tz}ATP** and NMN were treated with a recombinant human NMNAT-1 (Tris pH 7.8, 37°C). Adding an inorganic pyrophosphatase was essential to prevent product inhibition of the transferase[17] and facilitate a quantitative reaction as assessed by HPLC (Figure 1b). This enzymatic transformation was followed in real time by monitoring the emission intensity at 410 nm (λ_{ex} : 330 nm). The conversion of **^{tz}ATP** into **$N^{tz}AD^+$** was accompanied by a significant decrease in emission intensity over a 30 min window (Figure 1c). The in situ formed **$N^{tz}AD^+$** was then converted to the corresponding **N^{tz}ADH** over 10 min by the addition of ADH and ethanol to the NMNAT-1-mediated reaction mixture. The reduction of **$N^{tz}AD^+$** by ADH showed a further decrease in emission intensity comparable to the one observed for the enzymatic conversion of **^{tz}ATP** into **$N^{tz}AD^+$** (Figure 1c). Final addition of excess acetaldehyde triggered a nearly instantaneous enzymatic oxidation of **N^{tz}ADH**, recovering 80% of the original emission signal of **$N^{tz}AD^+$** (Figure 1c).

Steady-state absorption and emission spectra recorded at the end (plateau) of each enzymatic transformation show that the conversion of **^{tz}ATP** to **$N^{tz}AD^+$** has little or no effect on the absorption spectra of the **^{tz}A** chromophore but does lead to diminished fluorescence (Figure 1d). Further emission quenching is seen upon reduction of **$N^{tz}AD^+$** to **N^{tz}ADH**, along with an increase in the optical density at 333 nm, consistent with a reduced nicotinamide moiety.[18] All enzymatic reactions were also monitored by HPLC, corroborating the real-time fluorescence monitoring (Figure 1b). These observations illustrate the high photophysical responsiveness of the emissive **^{tz}A**-based cofactors. Despite the complexity of the reaction mixtures, which contain the buffered enzymes, substrates, and cofactors, resolved absorption and emission bands facilitate the spectral monitoring of the coupled processes. As a control, the same enzymatic cycle was performed with **^{tz}ATP** as the substrate, but without NMNAT-1. Absorption and emission measurements showed little to no change, thus indicating no conversion of **^{tz}ATP** over time (Figure S1).[15]

While numerous cellular processes utilize the NAD^+/NADH couple, several metabolic reactions exclusively use the $NADP^+$/NADPH pair, the corresponding monophosphorylated variant.[19] Natively, NAD^+ kinase (NADK) catalyzes the transfer of a phosphate group from ATP to the 2′-hydroxyl group on the adenosine's D-ribose moiety of NAD^+, while certain bacterial NADKs can also use poly-phosphate minerals as a phosphate source.[20] To critically assess the suitability of **$N^{tz}AD^+$** as a substrate, it was treated with *B. subtilis* NADK and ATP.[21] While phosphorylation was indeed observed (Figure 2), buildup of the newly synthesized **$N^{tz}ADP^+$** resulted in product inhibition, as expected.[20b] The kinase-mediated reaction was therefore coupled to a second enzymatic transformation, utilizing the in situ generated phosphorylated cofactor. Thus excess *S. cerevisiae* glucose-6-phosphate

dehydrogenase (G6PDH), an enzyme responsible for the oxidation of glucose-6-phosphate (G6P),[22] was added to the reaction mixture. The newly formed **N^{tz}ADP^{+}** was indeed converted to **N^{tz}ADPH** via G6PDH, as supported by HPLC and HRMS (see Figure 2d, and Figures S5 and S6). Importantly, NADK can also utilize **^{tz}ATP** as the phosphate donor for converting **N^{tz}AD^{+}** to **N^{tz}ADP^{+}**, thus illustrating a native enzymatic reaction, which utilizes two distinct synthetic cofactors (Figure S4).[15]

The NADK-mediated phosphorylation of **N^{tz}AD^{+}** to **N^{tz}ADP^{+}** is predictably photophysically "silent", while the following reduction of the in situ formed **N^{tz}ADP^{+}** to **N^{tz}ADPH** via G6PDH shows significant photophysical changes, comparable to those seen for **N^{tz}AD^{+}/N^{tz}ADH** (Figure 2a).[13b] Thus, over 3 hours, an increase in absorbance at 333 nm and a concomitant decrease of the emission intensity at 410 nm (λ_{ex}: 330 nm) are observed (Figure 2b). The overall reaction half-time, derived from the variation of emission spectra over time (Figure 2c), are 3.3 and 3.6×10^2 s for the native and the **^{tz}A**-based analogue nucleosides, respectively, assuming pseudo-first-order kinetics.[15] This process was also monitored in parallel by HPLC, showing 82% conversion for **N^{tz}AD^{+}**, compared to the 90% conversion when using native NAD^{+} (Figure 2d). Control experiments performed with **N^{tz}AD^{+}** in the absence of NADK yielded little to no variation in both the absorption and emission spectra (Figure S3).[15]

To assess the reverse reaction, **N^{tz}ADPH** was then subjected to treatment with *S. cerevisiae* glutathione reductase (GR). This enzyme converts oxidized glutathione (GSSG) to its reduced form (GSH) using NADPH as a cofactor, subsequently reducing it to NADP^{+}.[23] It is a significant player in controlling the cell's oxidative stress.[24] As seen in Figure 2b, this enzyme was able to instantly reduce GSSG to GSH with **N^{tz}ADPH**. Indeed, restoration of visible fluorescence (λ_{ex}: 330 nm, λ_{em}: 410 nm; Figure 2b) was instantaneously observed, demonstrating again the utility of our emissive analogues, where such enzymatic processes can be monitored by exclusive absorption and emission signal changes, despite the complexity and multicomponent nature of the reaction mixtures.

In this study we demonstrated that **N^{tz}AD^{+}** could be enzymatically prepared from **^{tz}ATP** within minutes.[14] While the previously reported syntheses of NAD^{+} analogues, using activated nicotinamide mononucleotide, are reported to take on the order of days,[25] we have shown that our emissive NAD^{+} analogue may be enzymatically formed significantly faster and subsequently used for further enzymatic processes. **N^{tz}AD^{+}**, the nicotinamide-containing emissive cofactor, can then be enzymatically converted to **N^{tz}ADP^{+}**, which displays responsive photophysical features, allowing one to monitor enzymatic processes in real time by visible emission spectroscopy. We then illustrated that those synthetic emissive analogues could replace the native cofactors in complex enzymatic networks. Furthermore, we finally showed that **N^{tz}ADPH**, the enzymatically generated reduced form, can be converted back to **N^{tz}ADP^{+}** using judiciously selected enzymatic reactions. Conveniently, this **N^{tz}ADP^{+}/N^{tz}ADPH** couple showed a photophysical behavior complementary to the photophysical changes seen for the native NADP^{+} and NADPH.[13b]

Our observations suggest that synthetic **^{tz}A**-based cofactors are faithful surrogates of the analogous native adenosine-based coenzymes. Enzymatic pathways for their preparation,

which could be employed in any laboratory using commercially available enzymes, have been demonstrated. Of particular significance is the ability to perform such enzyme-mediated reactions in a sequential manner, exploiting in situ generated substrates and cofactors. Coupled to their useful photophysical characteristics, which include visible emission and responsive behavior, most transformations can be fluorescently monitored in real time by common spectrometers. We submit that such synthetic fluorescent cofactors can find utility as tools for monitoring redox reactions in in vitro biophysical and discovery assays or potentially in living systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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14. We will provide samples of ^{tz}ATP upon request.
15. See the Supporting Information for additional experimental procedures and data.
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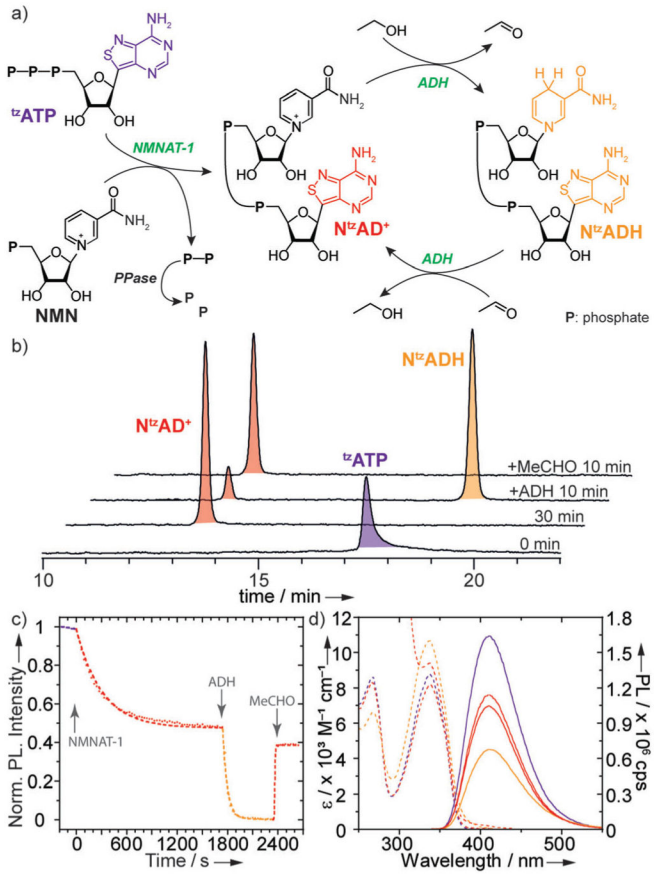


Figure 1.

a) Enzymatic cycle for **N^{tz}AD^{+}** generation, consumption, and regeneration with NMNAT-1 and ADH. b) HPLC traces monitored at 330 nm for the enzymatic conversions of **^{tz}ATP** (purple) to **N^{tz}AD^{+}** (red) and **N^{tz}ADH** (orange), before the addition of NMNAT-1, after 30 min, 10 min after addition of ADH, and 10 min after addition of acetaldehyde. c) Enzymatic transfer of NMN to **^{tz}ATP** (red) by NMNAT-1 to form **N^{tz}AD^{+}**, followed by oxidation of ethanol to acetaldehyde with ADH, generating **N^{tz}ADH** (orange), succeeded by a reverse enzymatic reaction with an excess of acetaldehyde to regenerate **N^{tz}AD^{+}** (red), monitored by real-time emission at 410 nm (λ_{ex} : 330 nm). d) Steady-state absorption (dashed line) and emission (solid line) spectra of **^{tz}ATP** (purple), in situ generated **N^{tz}AD^{+}** (red), **N^{tz}ADH** (orange), and regenerated **N^{tz}AD^{+}**, recorded at the end of each time-based enzymatic reaction.

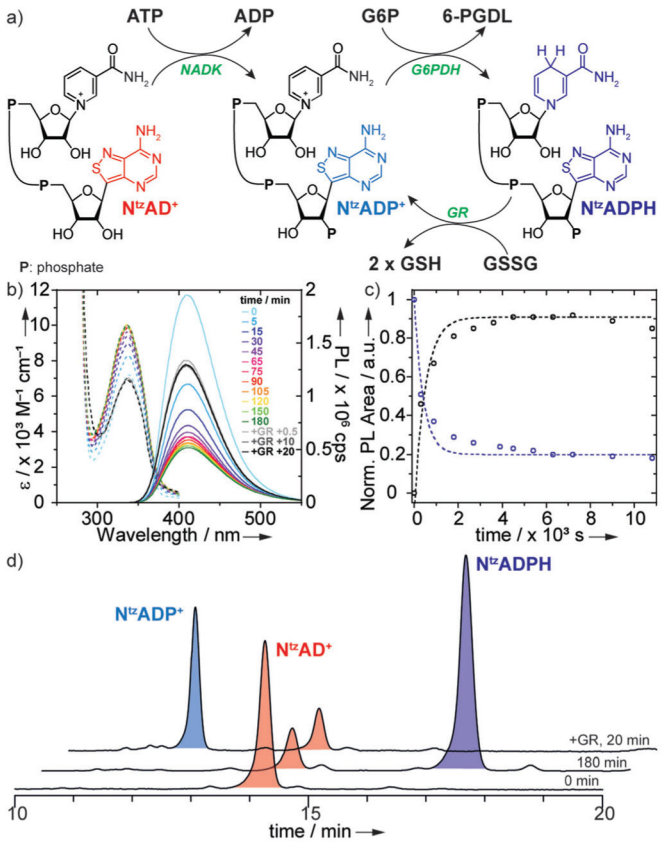
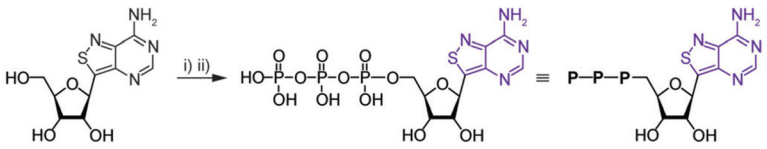


Figure 2.

a) Enzymatic cycle for **N^{tz}AD^{+}** phosphorylation by NADK followed by G6PDH-mediated reduction of **N^{tz}ADP^{+}** to **N^{tz}ADPH** and final reoxidation of the latter by GR with GSSG as substrate. b) Time-dependent steady-state absorption and emission spectra for the enzymatic conversion of **N^{tz}AD^{+}** to **N^{tz}ADPH** from 0 (cyan) to 180 minutes (green) and final oxidative regeneration of **N^{tz}ADP^{+}** by GR (grey and black). c) Normalized emission area over time for the conversion of **N^{tz}AD^{+}** to **N^{tz}ADPH** (blue, λ_{ex} : 330 nm) and NAD^{+} to NADPH (black, λ_{ex} : 335 nm). d) HPLC traces monitored at 330 nm for the enzymatic conversion of **N^{tz}AD^{+}** (red) to **N^{tz}ADPH** (blue), before the addition of NADK, after 180 min and after GR-mediated oxidation of **N^{tz}ADPH** to **N^{tz}ADP^{+}** (cyan).



Scheme 1.

Synthesis of **^{tz}ATP**. i) $POCl_3$, $(MeO)_3PO$, 0 °C to rt, 2 h; ii) tributylammonium pyrophosphate, Bu_3N, 0 °C to rt, 2 h, 20%.