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A novel fluorescent probe for NAD-consuming enzymes†

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A novel, fluorescent NAD derivative is processed as substrate by three different NAD-consuming enzymes. The new probe has been used to monitor enzymatic activity in a continuous format by changes in fluorescence and, in one case, to directly visualize alternative reaction pathways.

Nicotinamide adenine dinucleotide **1** (NAD, Fig. 1) is an essential cofactor found in all living cells. NAD-dependent enzymes are involved in many fundamental biological processes, including energy metabolism, cell signaling, gene expression and epigenetic control.[1] They can be grouped into redox enzymes, which draw on the reversible reduction/oxidation of the nicotinamide fragment,[2] and NAD-consuming enzymes such as glycohydrolases, pyrophosphatases and ADP-ribosyl transferases, which cleave one of the high-energy bonds in NAD (*i.e.* the pyrophosphate or *N*-glycosidic bond).[3] Individual NAD-consuming enzymes have been identified as potent regulators of key cellular functions such as calcium homeostasis and DNA repair.[3,4] Structural analogues of NAD are therefore highly sought after as biochemical tools to study these enzymes and processes.

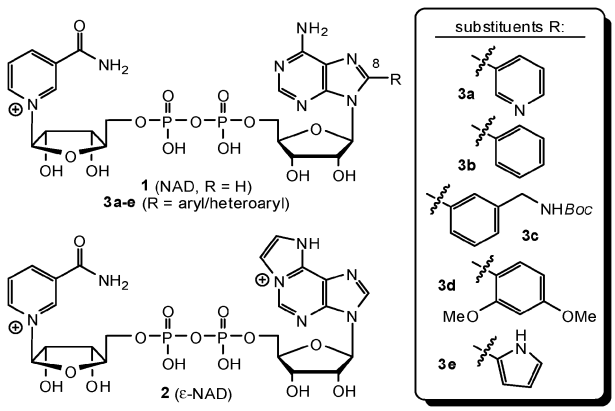

Fig. 1 Chemical structures of natural NAD **1**, 1,N^6-ethenoadenine dinucleotide **2**, and NAD derivatives **3a–e** developed in this study.

To date, structural modifications of NAD have focused mostly on the nicotinamide riboside and pyrophosphate moieties,[5] while modifications on the adenine ring are relatively rare.[6,7] One important example is 1,N^6-etheno NAD **2** (ε-NAD, Fig. 1), a fluorescent NAD derivative that has found very wide application in biochemical assays for NAD-consuming enzymes.[7,8] These assays are based on the fluorescence emission of the 1,N^6-etheno adenosine fragment, which in intact ε-NAD **2** is internally quenched by the nicotinamide moiety.[7,8] Chemical or enzymatic removal of the nicotinamide leads to a fluorescence signal that can be used to follow this reaction. Although introduced in the early 1970s, **2** and its analogues have remained as the only fluorophores available for such applications to date. However, **2** is an unsuitable substrate for a number of important NAD-consuming enzymes,[9] and its chemical structure does not allow optimization of its enzymological or fluorescence properties.

Herein, we describe a new family of NAD fluorophores with a fluorogenic substituent in position 8 of the adenine base and tunable fluorescence properties (Fig. 1, **3a–e**). We show that the derivative with the most interesting fluorescence characteristics, **3e**, is recognized as a substrate by three different NAD-consuming enzymes and can be used to monitor enzyme activity in real time. The new probe has unique practical utility as it allows, for the first time, direct visualisation of the different reaction pathways of a multi-functional ADP-ribosyl cyclase involved in cell signaling.

The 8-substituted NAD derivatives **3a–e** were prepared from the corresponding adenosine monophosphate (AMP) building blocks **5a–e** (Scheme 1).† The fluorogenic substituent was introduced at position 8 of AMP under aqueous Suzuki–Miyaura conditions,[6c,10] followed by $MnCl_2$-catalyzed pyrophosphate bond formation.[11] Depending on the nature of the 8-substituent, the NAD derivatives **3a–e** showed variable fluorescence characteristics (Fig. S1 and Table S1, ESI†) with particularly pronounced differences in the fluorescence quantum yields Φ of individual analogues (Table 1). Interestingly, fluorescence in this series was pH-dependent, with a sharp increase in emission between pH 3–6 for all dinucleotides apart from **3d** (Fig. S2, ESI†). Above pH 6 fluorescence remained stable, which is important with a view towards potential biological applications, as the pH working range of several NAD-dependent enzymes is between pH 7–8.

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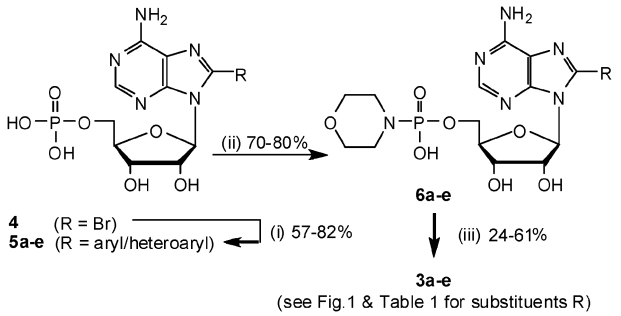

Scheme 1 *Reagents and conditions:* (i) Na_2PdCl_4, TPPTS, R-$B(OH)_2$, K_2CO_3, H_2O, Δ, 1–24 h; (ii) morpholine, dipyridyldisulfide, Ph_3P, DMSO, rt, 2 h; (iii) β-NMN, dry $MgSO_4$, $MnCl_2$ 0.2 M in formamide, rt, 24 h.

Table 1 Quantum yields of NAD (**3**) and AMP (**5**) derivatives in water

Cmpd	R	Φ_3	Φ_5	Φ_5/Φ_3
2	*Not applicable*	0.028–0.09[a]	0.56–0.59[a]	6–20
a[b]	3-Pyridinyl	0.003	0.003	1
b[b]	Phenyl	0.207	0.669	~3
c[b]	3-(*Boc*-aminomethyl)phenyl	0.150	0.888	~6
d[b]	2,4-*DMT*-pyrimidinyl	0.003	0.021	7
e[b]	2-Pyrrolyl	0.005	0.231	~46

[a] Values from ref. 7, 8 and 12*b*. [b] **3** or **5**, for scaffolds see Scheme 1.

Importantly, when we compared the fluorescence emission of AMP/NAD pairs with the same 8-substituent, we found that Φ was generally markedly higher for the AMP derivative (Table 1). As previously described for **2**,[7,8] this observation can be attributed to intramolecular fluorescence quenching in the case of the intact NAD derivatives due to the interaction of the nicotinamide and substituted adenine rings. To test this hypothesis, we subjected NAD derivatives **3a–e** to chemical hydrolysis under basic conditions, and followed these reactions by fluorimetry. As expected, fluorescence emission increased significantly upon hydrolytic removal of the nicotinamide ring (Fig. S3 and S4, ESI†), due to the abolition of the internal quenching effect. The hydrolytic degradation into the corresponding ADPR (adenosine diphosphate ribose) and nicotinamide was also confirmed in HPLC control experiments with **3e** (Fig. S5, ESI†). This NAD derivative showed by far the strongest fluorescence increase, as expected from the considerable difference in quantum yields between **3e** and **5e** (Table 1). Significantly, while for most 8-substituted AMP/NAD pairs the fluorescence emission ratio Φ_5/Φ_3 is in the same range as for ε-AMP/ε-NAD, for **5e**/**3e** this ratio is up to 7-fold greater than reported values for $\Phi_{\varepsilon\text{-AMP}}/\Phi_{\varepsilon\text{-NAD}}$. We speculated that this could allow for a more sensitive fluorescence-based assay of NAD-consuming enzymes than is possible with **2**. **3e** would be particularly attractive for such applications as its λ_{ex} and λ_{em} do not interfere with the intrinsic fluorescence of most proteins (Fig. S6, ESI†).

In order to assess the suitability of **3e** as a non-natural substrate for NAD-consuming enzymes, we chose three commercially available enzymes—nucleotide pyrophosphatase (NPP) from *Crotalus adamanteus* venom,[12] NAD-glycohydrolase (NGH) from porcine brain,[13] and ADP-ribosyl cyclase (ADPRC) from *Aplysia californica*[14]—which catalyse different covalent modifications of NAD (Scheme S1, ESI†). Following the

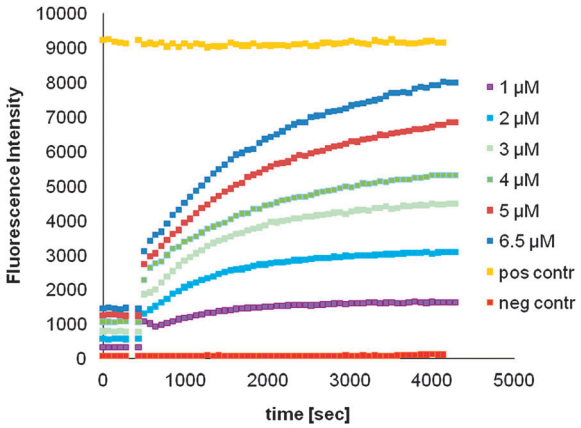

Fig. 2 Fluorimetric nucleotide pyrophosphatase assay of **3e**. *Conditions*: 0.007 U mL^{-1} enzyme, **3e** (0.2–6.5 µM) in 10 mM $MgCl_2$, 50 mM Tris/HCl (pH 8), 30 °C, λ_{ex} = 300 nm, λ_{em} = 410 nm, gain 15%. Enzyme addition at 400 s. Positive control (3 µM **5e**) and negative control (buffer) included.

consumption of **3e** and the formation of the respective reaction product for each enzyme by HPLC, we found that, pleasingly, all three enzymes use **3e** as a substrate (Fig. S7–S9, ESI†). Next, we investigated if the progress of these enzyme reactions could be monitored by fluorimetry. As expected, the consumption of **3e** by NPP led to a progressive increase in fluorescence which could be followed in a continuous assay format (Fig. 2). Similar results were obtained with NGH (Fig. S10, ESI†), although the limited aqueous solubility of this membrane-bound enzyme[13] complicated the *in situ* monitoring of the reaction and necessitated an additional pipetting step.

With NPP, we used the continuous assay for the enzymological characterization of **3e**. After confirming the linearity of the fluorescence response across a range of concentrations for both **3e** and its enzymatic product **5e**, we found that the initial reaction velocities were proportional to the enzyme concentration (Fig. S11 and S12, ESI†). Having established a suitable enzyme/substrate ratio, the substrate concentration was varied to generate a saturation curve for the determination of the K_m value of **3e**. The variation of the initial velocity with the concentration of **3e** was well described by the Michaelis–Menten equation and Hanes–Woolf plots, resulting in K_m of 6.22 ± 3.08 µM and v_{max} of 0.0530 ± 0.0140 µmol min^{-1} mg^{-1} (Fig. S13, ESI†). This K_m value for **3e** is comparable to literature values for NAD with several pyrophosphatases.[12] In contrast, the frequently used fluorophore **2** and its derivative ε-PdAD have often higher K_m values (Table S2, ESI†).[12] This may be due to interference of the etheno bridge in **2** with hydrogen bonding of the pyrimidine ring at the cofactor binding site. Substitution in position 8 in **3e**, on the other hand, does not perturb hydrogen bond formation. These results therefore suggest that, at least for NPP, the new fluorophore **3e** is a closer model of the natural cofactor NAD than **2**.

Finally, we studied the behavior of **3e** towards the multifunctional enzyme ADP-ribosyl cyclase (ADPRC).[14] ADPRC catalyzes primarily the cyclization of NAD at the N1 position to give N1-cADPR (cyclic adenosine diphosphate ribose), but also the hydrolysis of N1-cADPR into ADPR (Scheme S2, ESI†).[14a] N1-cADPR is an important 2nd messenger and mediator of

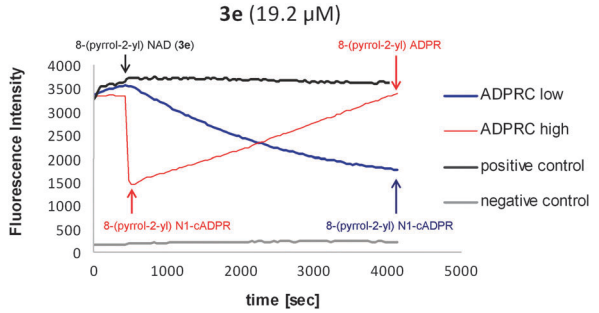

Fig. 3 Fluorimetric ADP-ribosyl cyclase assay of **3e**. *Conditions*: **3e** (19.2 μM), ADPRC (blue line: 0.025 U mL^{-1}, red line: 1.75 U mL^{-1}), HEPES buffer (50 mM, pH 7.4), 25 °C, λ_{ex} 300 nm, λ_{em} 410 nm, gain 15%. Enzyme addition at 780 s. Positive control: **3e** only; negative control: buffer.

IP_3-independent calcium signaling,[15] and structural analogues of N1-cADPR are sought after as biological tools.[16] Evidently, the natural N1-cyclization of ADPRC is not possible in the case of the existing fluorophore **2**, due to the presence of the etheno bridge. Instead, **2** redirects ADPRC activity, giving rise to a non-natural, fluorescent derivative cyclized at N7.[14b]

Upon incubation of **3e** at either 19.2 μM or 9.6 μM with ADPRC at 0.025 U mL^{-1}, we observed a decrease in fluorescence (Fig. 3 and Fig. S14, ESI†, blue lines). This result suggests that, like NAD, **3e** is indeed cyclized at N1, giving rise to 8-(pyrrol-2-yl) N1-cADPR, as cyclization at N7 would be expected to lead to an increase, not a decrease in fluorescence. Using this decrease in fluorescence to determine the enzymological parameters of **3e** for the cyclase activity of ADPRC, we obtained K_m of 74 ± 28 μM and v_{max} of 21.80 ± 4.11 μmol min^{-1} mg^{-1} (Fig. S15–S17, ESI†). This K_m value of **3e** is in the same range as literature values for the natural substrate NAD, in contrast to **2** and other N7-cyclised dinucleotide substrates of ADPRC, for which significantly lower K_m values have been reported (Table S3, ESI†).

Interestingly, when the same experiments were carried out at a higher concentration of ADPRC, fluorescence emission increased again after the initial drop (Fig. 3 and Fig. S14, ESI†, red lines). It is known that the N1-cADPR-hydrolase activity of ADPRC is only unmasked at high enzyme concentrations.[14a] It therefore appears that under these conditions, ADPRC initially cyclizes **3e** into 8-pyrrolyl N1-cADPR, before hydrolysing the latter into the linear, and fluorescent, 8-pyrrolyl ADPR (Scheme S2, ESI†). This interpretation is in keeping with previous mechanistic studies on the different activities of ADPRC.[14a] Thus, the new fluorophore **3e** allows, for the first time, the direct visualization of the different reaction pathways of ADPRC, which makes it a unique tool for biological studies on this enzyme and its role in cell signalling.

In summary, we have developed a new type of fluorescent NAD derivative with a fluorogenic substituent in position 8 of the adenine base. We show that a specific analogue in this series, **3e**, is recognized as a substrate by three different NAD-dependent enzymes. Furthermore, **3e** can be used to monitor enzyme activity in continuous form and, in the case of ADPRC, to visualize different reaction pathways. Compared to the existing probe **2**, the new fluorophore offers significant advantages in terms of its enzymological and fluorescence properties, and the breadth of its applicability is the subject of ongoing studies.

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