ADP-Ribosylation

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Chain-Terminating and Clickable NAD$^+$ Analogues for Labeling the Target Proteins of ADP-Ribosyltransferases**

*Yan Wang, Daniel Rösner, Magdalena Grzywa, and Andreas Marx**

Abstract: *ADP-ribosyltransferases (ARTs) use NAD$^+$ as a substrate and play important roles in numerous biological processes, such as the DNA damage response and cell cycle regulation, by transferring multiple ADP-ribose units onto target proteins to form poly(ADP-ribose) (PAR) chains of variable sizes. Efforts to identify direct targets of PARylation, as well as the specific ADP-ribose acceptor sites, must all tackle the complexity of PAR. Herein, we report new NAD$^+$ analogues that are efficiently processed by wild-type ARTs and lead to chain termination owing to a lack of the required hydroxy group, thereby significantly reducing the complexity of the protein modification. Due to the presence of an alkyne group, these NAD$^+$ analogues allow subsequent manipulations by click chemistry for labeling with dyes or affinity markers. This study provides insight into the substrate scope of ARTs and might pave the way for the further developments of chemical tools for investigating PAR metabolism.*

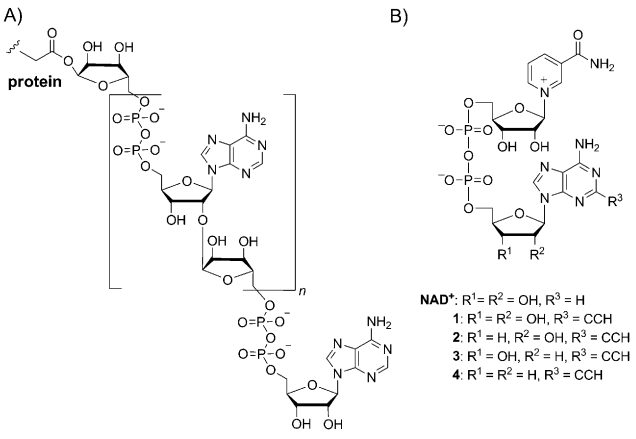

Figure 1. The structures of poly(ADP-ribose) (A), and NAD$^+$ and the NAD$^+$ analogues developed and employed in this study (B).

Based on its diverse functions, nicotinamide adenine dinucleotide (NAD$^+$) is well known to participate in a variety of cellular processes, such as redox metabolism, signaling pathways, and posttranslational modifications. In 1963, *Chambon* et al. reported for the first time the formation of a nucleic acid like polymer derived from NAD$^+$, namely poly(ADP-ribose)[1] (PAR; Figure 1 A). ADP-ribosylation is a reversible posttranslational modification of proteins and catalyzed by a family of enzymes termed ADP-ribosyltransferases (ARTs),[2] with ADP-ribosyltransferase diphtheria toxin-like 1 (ARTD1, formerly known as PARP-1) as the best described member. ARTs play important roles in a wide range of biological processes, including DNA repair, maintenance of genomic stability,[3–5] and transcriptional regulation.[6,7] In the human genome, 22 different genes that contain an ADP-ribosyltransferase catalytic domain have been identified.[8] ADP-ribosylation comprises the transfer of single or multiple ADP-ribose moieties from NAD$^+$ to specific amino acid residues on a target protein, thereby leading to mono-(ADP-ribos)ylation or poly(ADP-ribos)ylation. In this process, covalent transfer onto glutamic acid, aspartic acid, or lysine residues of target proteins is described.[9]

Successive transfer reactions onto the protein–mono-(ADP-ribosyl) adduct, and subsequently onto the nascent chain of several covalently linked ADP-ribosyl units, are the basis for the formation of PAR, which consists of up to 200 ADP-ribose units coupled through a 2′-*O*-α-D-ribofuranosyl-adenosine diphosphate backbone.[10,11]

Efforts to identify direct targets of ARTs, as well as the specific ADP-ribose acceptor sites, must all tackle the complexity of PAR, which complicates subsequent analysis. Current approaches harness mutant ARTs that catalyze only mono(ADP-ribos)ylation;[12] make use of protein-based or chemical approaches for enrichment or to cleave PAR chains from the substrate, thereby resulting in labeling;[13] or use chemically modified NAD$^+$ analogues.[12b,14]

Herein, we report new NAD$^+$ analogues that are efficiently incorporated by wild-type ARTs and a) lead to PAR chain termination owing to a lack of the required hydroxy group and b) bear an affinity tag that will allow subsequent manipulations such as labeling and sample enrichment (Figure 1 B). Since little is known about the substrate scope of ARTs,[14,15] we developed four NAD$^+$ analogues (**1**–**4**, Figure 1 B) with systematic deoxygenation of the hydroxy groups of adenosine and an alkyne group on the nucleobase. These modifications enabled us to investigate the influence of the respective OH group on ART (i.e., ARTD1) activity.

The synthesis of the analogues **1**–**4** was conducted by first synthesizing the modified adenosine cores **5**–**8**, which bear an iodine atom at position 2 (Scheme 1 and Schemes S1, S2 in the Supporting Information). The alkyne function was then introduced with the Sonogashira reaction[16] to yield **9**–**12**, which were subsequently converted into the monophosphates

[*] Y. Wang, D. Rösner, M. Grzywa, Prof. Dr. A. Marx
Fachbereich Chemie, Universität Konstanz
Universitätsstrasse 10, 78457 Konstanz (Germany)
E-mail: andreas.marx@uni-konstanz.de
Homepage: http://www.uni-konstanz.de/chemie/~agmarx/

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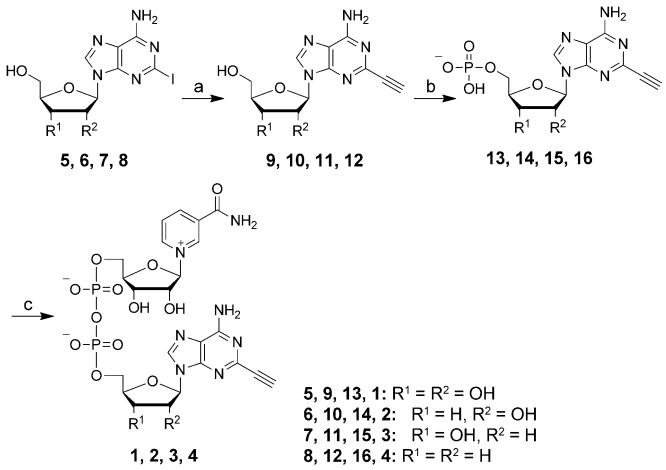

Scheme 1. a) 1) CuI, trimethylsilylacetylene, $Pd(PPh_3)_2Cl_2$, Et_3N, DMF, RT, 16 h; 2) NH_3/MeOH, RT, 1.5 h, 45% for **9** in 2 steps, 80% for **10** in 2 steps, 81% for **11** in 2 steps, 82% for **12** in 2 steps. b) proton sponge, $POCl_3$, trimethyl phosphate, 0 °C, 5 h, 40% for **13**, 47% for **14**, 44% for **15**, 61% for **16**. c) β-NAM, CDI, triethylamine, DMF, RT. 96 h, 20% for **1**, 23% for **2**, 25% for **3** and **4**. β-NAM = β-Nicotinamide monophosphate, CDI = *N*,*N*′-Carbonyldiimidazole.

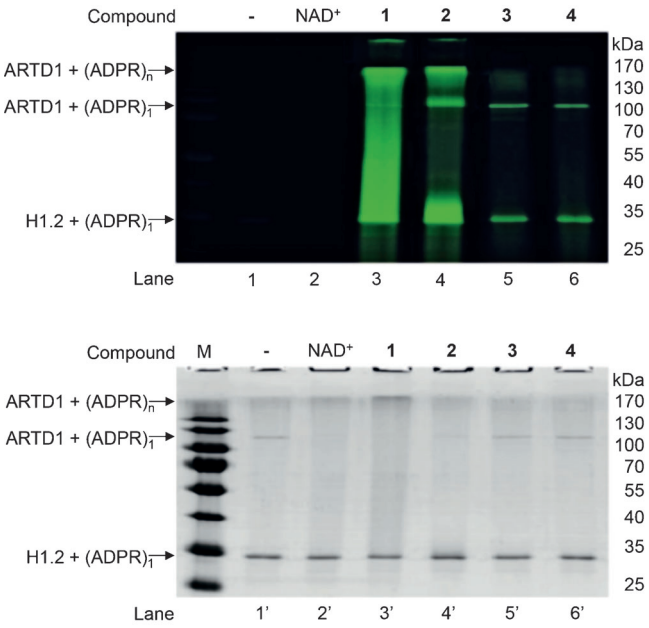

Figure 2. Modification of histone H1.2 and ARTD1 with the NAD^+ analogues. The top panel shows the sulfo-Cy5 fluorescence and the bottom panel shows the same gel stained with Coomassie Blue. Lanes 1 and 1′: negative control (ADP-ribosylation without oligonucleotide duplex; for further experiments see Figure S2); lanes 2 and 2′: positive control (ADP-ribosylation with natural NAD^+); lanes 3 and 3′: ADP-ribosylation with **1**; lanes 4 and 4′: with **2**; lanes 5 and 5′: with **3**; lanes 6 and 6′: with **4**. The total concentration of all NAD^+ analogues was 1 mM.

13–**16** through phosphorylation[17] (Scheme 1). The respective monophosphates were subsequently converted into the NAD^+ analogues **1**–**4** by reaction with activated β-nicotinamide-monophosphate (Scheme 1).[18]

With NAD^+ analogues **1**–**4** in hand, we tested them in in vitro ADP-ribosylation assays with ARTD1, the best studied ARTD family member. During ADP-ribosylation, ARTD1 serves as its own acceptor[12] in a process known as auto(ADP-ribos)ylation, as well as modifying acceptor proteins in a trans(ADP-ribos)ylation process. We thus investigated the substrate scope of ARTD1 with regard to accepting **1**–**4** in auto(ADP-ribos)ylation (Figure S5 in the Supporting Information) and trans(ADP-ribos)ylation (Figure 2) reactions. In the trans(ADP-ribos)ylation assay, ARTD1 and histone H1.2, which is the main acceptor of ADP-ribose[19] and is modified by ARTD1 and ARTD3 in vitro,[20] were incubated with each of the NAD^+ analogues in the presence of an octameric oligonucleotide duplex that activates ARTD1.[21] Reaction with natural NAD^+ served as a positive control and for the negative control, the reaction was performed in the absence of the activating oligonucleotide duplex. After the enzymatic reaction, click chemistry was performed to conjugate a fluorescent dye, sulfo-Cy5-azide (Figure S1), to the alkyne-modified ADP-ribose units derived from the incorporation of the modified NAD^+ analogues. After removing unreacted sulfo-Cy5-azide, the reaction mixtures were analyzed by SDS-PAGE and the fluorescent labeling was detected and compared with the gel stained with Coomassie Blue.

As shown in Figure 2, all NAD^+ analogues are accepted for the trans(ADP-ribos)ylation of H1.2 by ARTD1 (Figure 2, lanes 3–6), with **1** and **2** showing the strongest fluorescent signals owing to the formation of longer PAR chains and the incorporation of multiple alkyne functionalities (Figure 2, lanes 3 and 4). With **1**, both, ARTD1 and H1.2 undergo extensive poly(ADP-ribos)ylation. Differences in the composition of the attached PAR led to variable migration propensity for the H1.2- and ARTD1-derived products (Figure 2, lanes 3 and 3′) in PAGE analysis. Interestingly, we found similar performance in the detection of PAR by using **1** and click chemistry, and conventional Western blots with an antibody (Figure S3). For **2**, we found that the absence of the 3″-OH group influences the formation of PAR (Figure 2, lane 4). Although this analogue was clearly incorporated (since the product could be stained by click reaction with a dye), the automodification product pattern was altered compared to the one obtained when **1** was used. This result suggests an unexpected participation of the 3″-OH group in ARTD1 catalysis. In contrast to the results obtained with **1** and **2**, the use of **3** and **4** prevents the formation of long PAR chains; these analogues thus act as chain terminators, as shown by the distinct fluorescent bands of labeled H1.2 and ARTD1 observed with **3** and **4** (Figure 2, lanes 5 and 6). Interestingly, when the 3″-OH group was provided with **3**, PAR assembly could not be rescued and a similar modification pattern to that seen with the dideoxy analogue **4** (Figure 2, lanes 5, 6) was observed. These findings confirm that the 2″-OH group is essential for PAR assembly by ARTD1.

Encouraged by these results, we investigated whether our approach enables biotin labeling of the (ADP-ribos)ylated products. Indeed, we were able to covalently connect biotin to the reaction products that were obtained through the use of the modified NAD^+ analogues **1**–**4** (see Figure S4). Interest-

ingly, similar results were obtained when auto(ADP-ribos)-ylation of ARTD1 was investigated (see Figure S5).

To determine whether the NAD^+ analogues are competitive substrates for ARTD1-catalyzed ADP-ribosylation, we tested **4** in a reaction competing with natural NAD^+. For this experiment, the enzymatic reaction was carried out as described above, but with different ratios of natural NAD^+ to **4**. The results clearly show that **4** is incorporated by ARTD1 in the presence of natural NAD^+ (Figure 3). Even

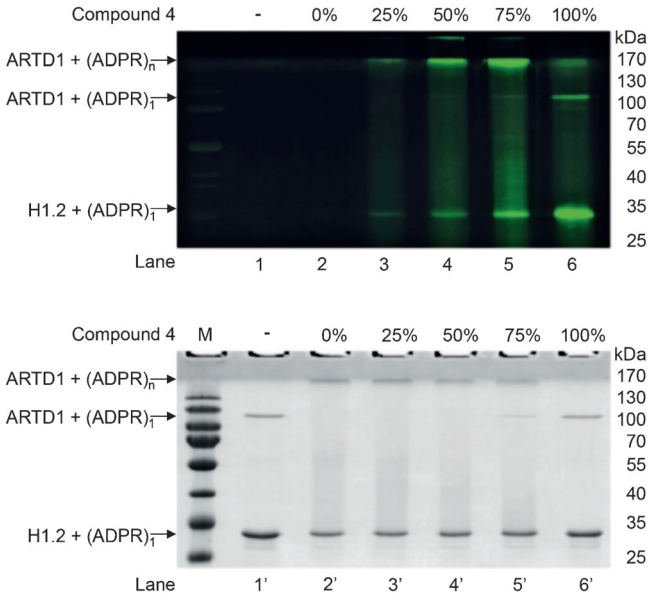

Figure 3. Labeling of ARTD1 and H1.2 with **4**. The top panel shows the sulfo-Cy5 fluorescence and the bottom panel shows the same gel stained with Coomassie Blue. Lanes 1 and 1′: negative control (trans-(ADP-ribos)ylation without oligonucleotide duplex); lanes 2 and 2′: positive control (trans(ADP-ribos)ylation with 100% natural NAD^+); lanes 3 and 3′: trans(ADP-ribos)ylation with 75% natural NAD^+ and 25% **4**; lanes 4 and 4′: with 50% natural NAD^+ and 50% **4**; lanes 5 and 5′: with 25% natural NAD^+ and 75% **4**; lanes 6 and 6′: 100% **4**. The total concentration of all NAD^+ analogues was 1 mM.

with only 25% of **4**, a fluorescent signal corresponding to the incorporation of the alkyne functionality was detected after the click reaction with sulfo-Cy5-azide (Figure 3, lanes 3–6). Interestingly, ADP-ribosylation of H1.2 by processing with **4** appears to take place at all employed concentrations of **4**. By contrast, ARTD1 ADP-ribosylation was only observed when **4** was exclusively employed. This indicates that **4** is more efficiently used by ARTD1 for the trans(ADP-ribos)ylation reaction of histone H1.2 than for auto(ADP-ribos)ylation (Figure 3, lanes 3–6). We found that analogues **1**–**3** are also incorporated by ARTD1 in the presence of natural NAD^+ (see Figure S6).

In Summary, we report the design and syntheses of four novel NAD^+ analogues, which were modified at the purine ring and in the number of hydroxy groups on the adenosine. All of the modified NAD^+ analogues (**1**–**4**) were applied in enzymatic studies and found to be efficient substrates for ARTD1 and were used to label ADP-ribosylated ARTD1 and H1.2. By the systematic modification of the hydroxy groups of adenosine, we explored the substrate scope of ARTD1 in terms of its dependence of the presence of a 2″- and 3″-OH group in the assembly of poly(ADP-ribose). Whereas **1** led to the formation of long PAR, **3** and **4** act as chain terminators when applied in auto- or trans(ADP-ribos)ylation reactions. This confirms the need for the 2″-OH for PAR formation and shows that the absence of this group cannot be rescued by the presence of a 3″-OH. Furthermore it was found that the 3″-OH group participates in the ADP-ribosylation of ARTD1 since the employment of **3** leads to less efficient PAR formation as compared to the reactions where an analogue with both 2″- and 3″-OH groups (**1**) was employed. We further found that the modifications carried by **4** turn this component into an efficient chain terminator of PAR synthesis that competes with natural NAD^+ in ADP-ribosylation reactions. This study provides insight into the substrate scope of ARTD1 and its catalytic mechanism for auto- and trans-(ADP-ribos)ylation. Furthermore, the herein described NAD^+ analogues **1**–**4** add to the currently limited toolbox of NAD^+ analogues for studying ADP-ribosylation processes. The chain-terminating analogues should facilitate the identification and analysis of protein targets of ADP-ribosylation in proteomics approaches, since first results of labeling histone H1.2 in the context of a cell lysate are very promising (see Figure S7), and should thus spur progress towards the development of new chemical tools to elucidate the metabolism of PAR.

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