Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

A Ribose-Functionalized NAD$^+$ with Versatile Activity for ADP-Ribosylation

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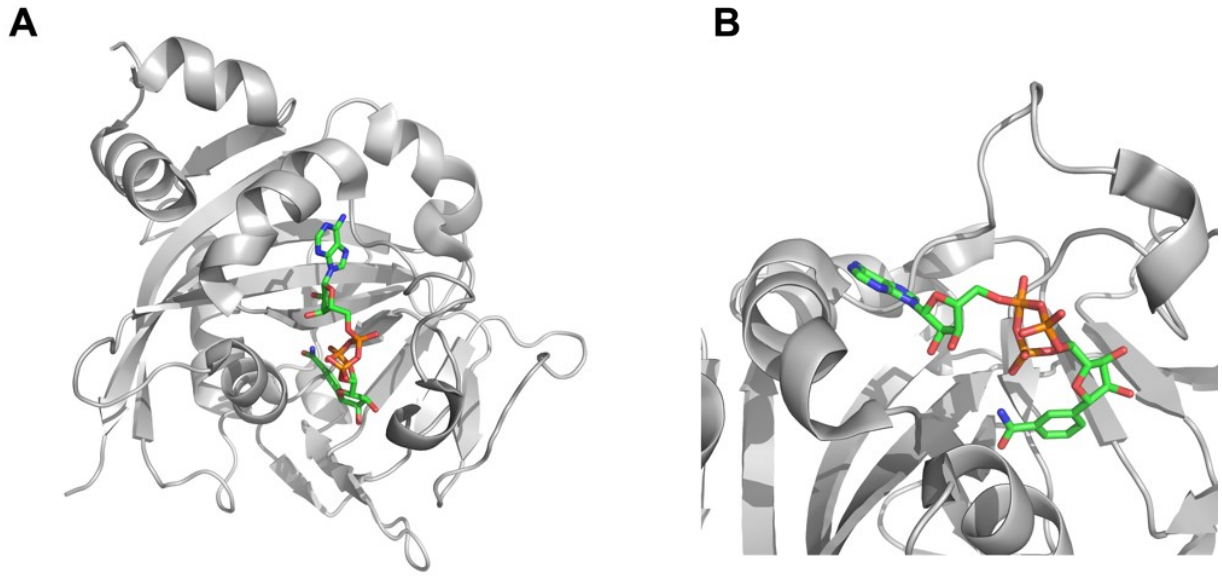
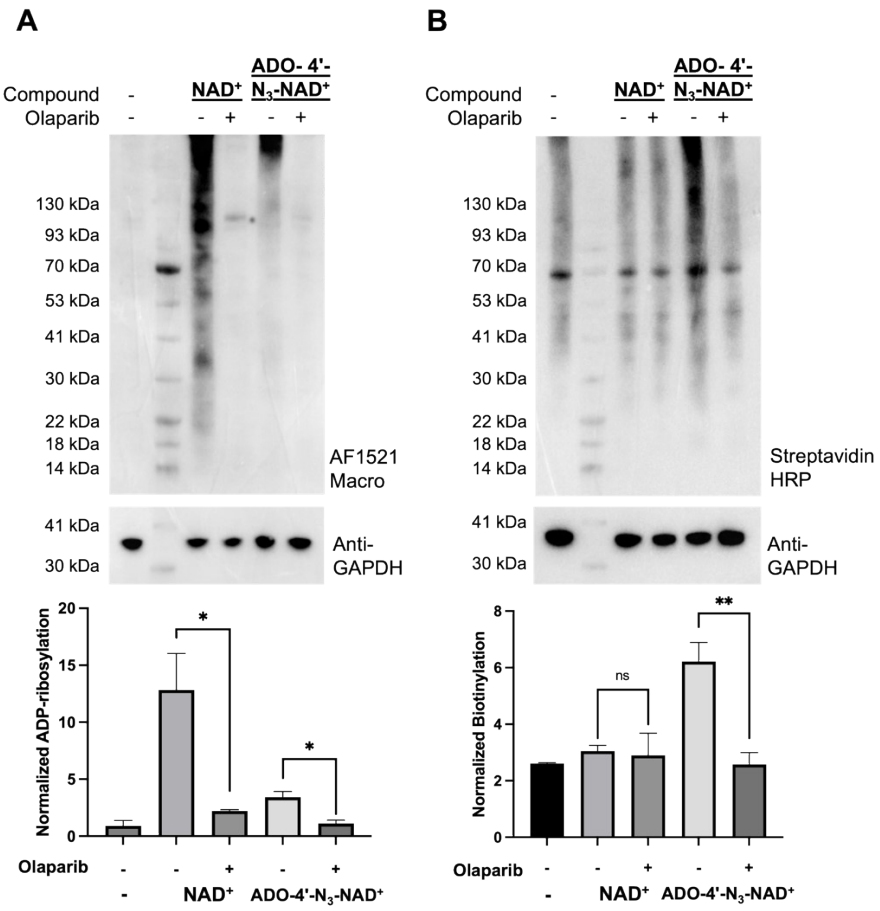
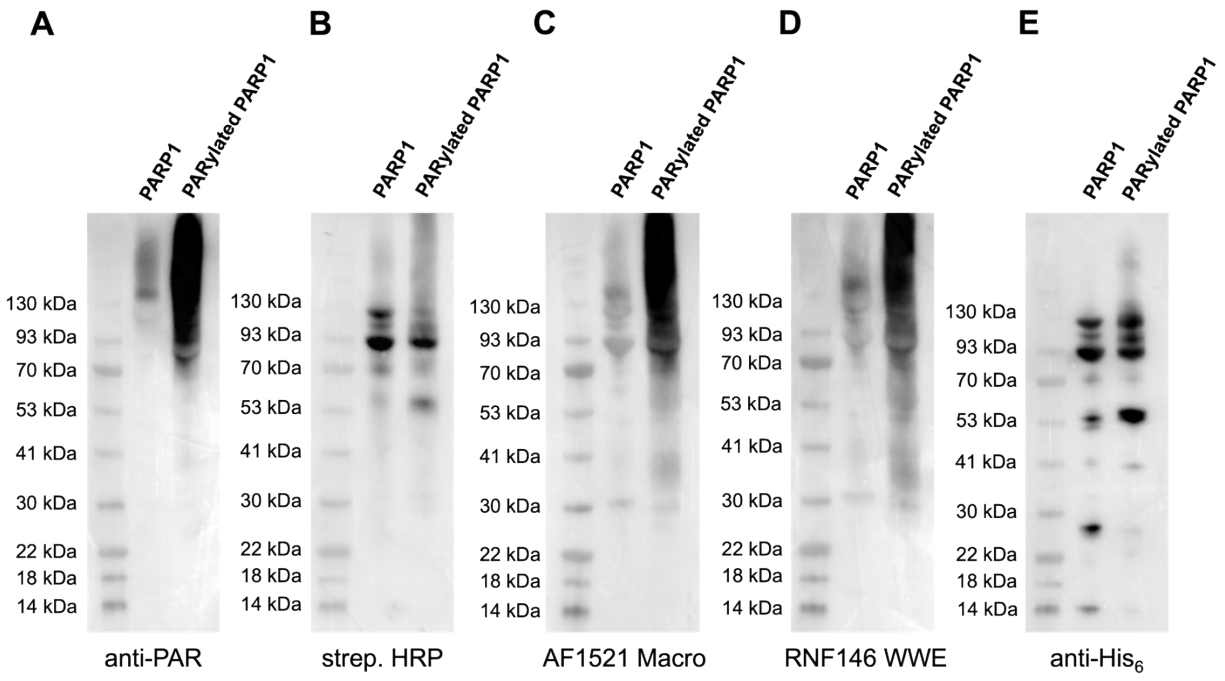
Supplemental Figures



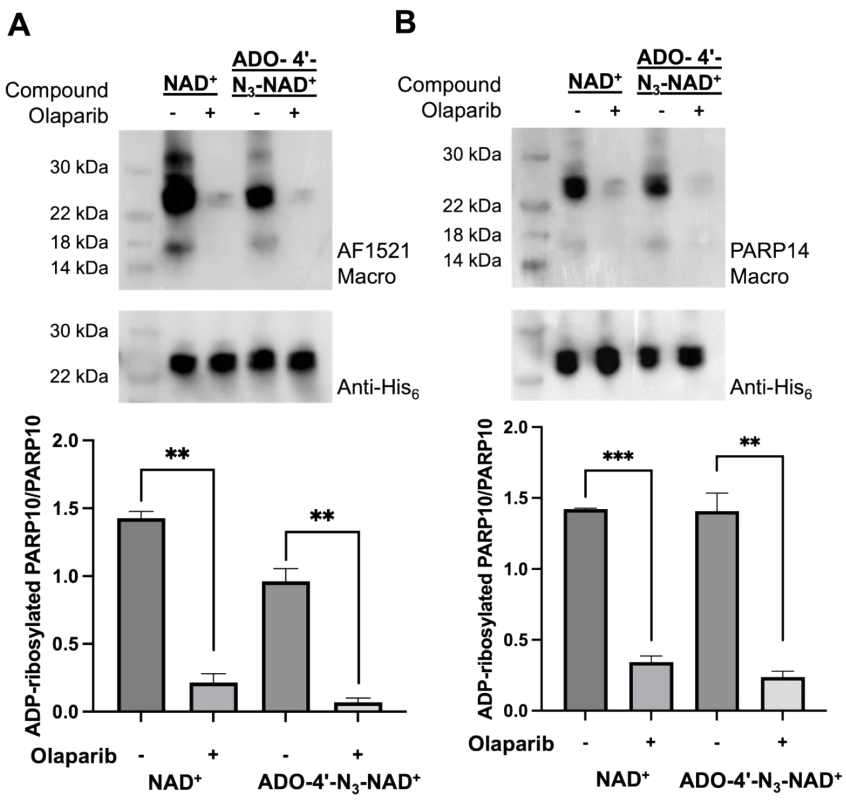
Supplementary Figure S1. The X-ray structure of human PARP1 bound to benzamide adenine dinucleotide (BAD) (PDB ID: 6BHV).[1] (A) Overall structure of human PARP1 in complex with BAD. (B) Active site of the complex of human PARP1 with BAD.



Supplementary Figure S2. Protein ADP-ribosylation for HEK293T cell lysates with NAD^+ or ADO-4′-N_3-NAD^+. (A) Detection of protein ADP-ribosylation by an AF1521 macrodomain. (B) Detection of protein ADP-ribosylation with a streptavidin-HRP conjugate after biotin labeling. Cell lysates were incubated with 600 μM NAD^+ or ADO-4′-N_3-NAD^+ with or without olaparib at room temperature overnight. Bottom panels: densitometric analysis of protein ADP-ribosylation normalized toanti-His_6 loading controls. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.



Supplementary Figure S3. Validation of PARylation-specific reader proteins. Non-modified human PARP1 and PARylated human PARP1 derived from NAD^+ were analyzed by immunoblots by (A) an anti-PAR monoclonal antibody, (B) a streptavidin-HRP conjugate after biotin labeling, (C) an AF1521 macrodomain, (D) a RNF146 WWE domain, and (E) an anti-His_6 antibody. Auto-modification of PARP1 with 300 μM NAD^+ was carried out at 30°C for 2 hours.



Supplementary Figure S4. Recognition of ADO-4′-N_3-NAD^+-based MARylation by reader proteins. Binding of MARylated PARP10 derived from NAD^+ or ADO-4′-N_3-NAD^+ by (A) AF1521 macrodomain and (B) PARP14 macrodomain was evaluated by immunoblots. Auto-MARylation of the catalytic domain of human PARP10 with 400 μM NAD^+ or ADO-4′-N_3-NAD^+ was carried out at room temperature overnight. Bottom panels: densitometric analysis of protein ADP-ribosylation normalized to anti-His_6 loading controls. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Experimental Methods

General materials and methods

Unless otherwise indicated, all procedures were performed under ambient conditions and reagents were used as received. The template for producing the DNA sequence encoding full-length human PARP2 with a C-terminal His_6-tag was a gift from Dr. Tom W. Muir (Addgene plasmid #111574).[2] Enzymes used in the cloning and generation of human PARP2 were purchased from New England Biolabs (MA, USA). Antibodies used for immunoblotting analyses were purchased from Thermo Fisher Scientific (MA, USA), Sigma Aldrich (MO, USA), Santa Cruz Biotechnology (TX, USA), or R&D Systems (MN, USA). All other reagents and materials are commercially available.

Bacterial expression and purification of human PARP1

The bacterial expression and purification of full-length human PARP1 was performed as previously described with minor modifications.[3–8] Sterilized LB broth (5 mL) containing 50 μg/mL kanamycin was inoculated under sterile conditions with BL21 (DE3) *Escherichia coli* transformed with a pET28a (+) vector expressing full-length human PARP1 with a C-terminal His_6-tag and cultured overnight at 37°C at 220 rpm. The overnight 5-mL culture was then transferred under sterile conditions into 1 L of sterilized LB broth containing 50 μg/mL kanamycin and grown to OD_{600} = 1.0 – 1.2 at 37 °C at 220 rpm. PARP1 expression was induced using a 500 mM stock of isopropyl β-*d*-1-thiogalactopyranoside (IPTG) and a 500 mM stock of $ZnSO_4$ to final concentrations of 500 μM each and grown overnight at 16 °C at 220 rpm. Cells were harvested by centrifugation at 4,000 rpm for 30 minutes at 4 °C and the supernatants were discarded. Cell pellets were resuspended in 25 mL cell lysis buffer (20 mM Tris pH 7.5, 500 mM NaCl, 20 mM imidazole,

1 mM beta-mercaptoethanol (β-ME), and 1 mM phenylmethylsulfonyl fluoride (PMSF)) and kept on ice. Cells were lysed by running through a pre-cooled French press at 25,000 psi three times. The cell debris was spun down by centrifugation at 15,000 rpm for 50 minutes at 4 °C and the supernatants were collected.

The collected supernatants were then filtered by passing through 0.45 μm membranes under vacuum and subsequently loaded into a gravity flow column with 1 mL of Ni-NTA agarose resin, which was washed with 15 mL equilibrium buffer (20 mM Tris pH 7.5, 500 mM NaCl, 20 mM imidazole, and 1 mM PMSF) before loading. An additional 15 mL equilibrium buffer was loaded following the cell lysate. Bound proteins were washed with 15 mL wash buffer (20 mM Tris pH 7.5, 500 mM NaCl, 40 mM imidazole, and 1 mM PMSF) before eluting with 15 mL elution buffer (20 mM Tris pH 7.5, 500 mM NaCl, 400 mM imidazole, and 1 mM PMSF). Onto a separate gravity flow column, 3 mL of heparin beads were loaded and washed with 15 mL water, followed by 15 mL heparin binding buffer (50 mM Tris pH 7.5, 250 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol (DTT), and 1 mM PMSF). The prepared heparin column was then loaded with a 1:1 mixture of eluted proteins and no-salt buffer (50 mM Tris pH 7.5, 1 mM EDTA, 0.1 mM DTT, 1 mM PMSF). Bound proteins were then washed of weakly bound proteins with 15 mL of a 1:1 mixture of heparin binding buffer and no-salt buffer, followed by 5 mL heparin binding buffer. Desired proteins were subsequently eluted with 15 mL heparin elution buffer (50 mM Tris pH 7.5, 1 M NaCl, 1 mM EDTA, 0.1 mM DTT, and 1 mM PMSF). β-ME was added to the eluted proteins solution to a final concentration of 1 mM before concentrating to 1 mL with a 30 kDa MWCO ultra-15 centrifugal filter unit. Purified PARP1 was examined by SDS-PAGE and a NanoDrop 2000C spectrophotometer, then aliquoted and flash-

frozen in liquid nitrogen for storage at -80 °C. Protein concentrations were determined by UV absorbance at 280 nm using an extinction coefficient of 1.05.

Cloning and bacterial expression and purification of human PARP2

Protein sequence of human PARP2 with a C-terminal His_6-tag : MAARRRRSTGGGRARALNESKRVNNGNTAPEDSSPAKKTRRCQRQESKKMPVAGGKA NKDRTEDKQDGMPGRSWASKRVSESVKALLLKGKAPVDPECTAKVGKAHVYCEGND VYDVMLNQTNLQFNNNKYYLIQLLEDDAQRNFSVWMRWGRVGKMGQHSLVACSGNL NKAKEIFQKKFLDKTKNNWEDREKFEKVPGKYDMLQMDYATNTQDEEETKKEESLKS PLKPESQLDLRVQELIKLICNVQAMEEMMMEMKYNTKKAPLGKLTVAQIKAGYQSLKK IEDCIRAGQHGRALMEACNEFYTRIPHDFGLRTPPLIRTQKELSEKIQLLEALGDIEIAIKL VKTELQSPEHPLDQHYRNLHCALRPLDHESYEFKVISQYLQSTHAPTHSDYTMTLLDLF EVEKDGEKEAFREDLHNRMLLWHGSRMSNWVGILSHGLRIAPPEAPITGYMFGKGIYFA DMSSKSANYCFASRLKNTGLLLLSEVALGQCNELLEANPKAEGLLQGKHSTKGLGKMA PSSAHFVTLNGSTVPLGPASDTGILNPDGYTLNYNEYIVYNPNQVRMRYLLKVQFNFLQ LWHHHHHH

The template for producing the DNA sequence encoding full-length human PARP2 with a C-terminal His_6-tag was obtained from Addgene (plasmid #111574).[2] To introduce compatible cut-sites within the backbone pET-28a (+) vector, the sequence was amplified through a polymerase chain reaction (PCR) using a designed forward primer (5'-GTGGTGCTCGAGTTATTAGTGATGGTGATGGTGATGCCAAAGTTG-3') and reverse primer (5'-TTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGCAGCC -3') purchased from Integrated DNA Technologies (IA, USA). The amplified full-length PARP2

PCR product and pET-28a (+) vector were digested by XhoI and XbaI restriction enzymes (New England Biolabs), purified using gel DNA recovery kits (Zymo Research, CA), and ligated using the T4 DNA ligase (New England Biolabs). The resulting bacterial expression vector was verified by DNA sequencing (Genewiz LLC, NJ) and subsequently used to transform BL21 (DE3) *E. coli* by electroporation.

To express and purify human PARP2, sterilized LB broth (5 mL) containing 50 ug/ml kanamycin was inoculated under sterile conditions using the transformed BL21 (DE3) *E. coli* and cultured overnight at 37 °C at 220 rpm. The 5-mL culture was then transferred under sterile conditions into 1 L sterilized terrific broth containing 50 ug/ml kanamycin and grown to OD_{600} = 1.2 – 1.4 at 37°C at 220 rpm. Expression of PARP2 was induced using IPTG to a final concentration of 500 μM and allowed to grow for 36 hours at 16 °C at 220 rpm. Cells were harvested by low-speed centrifugation at 4 °C, and the resulting cell pellets were resuspended in 25 mL cell lysis buffer (20 mM Tris pH 7.5, 500 mM NaCl, 20 mM imidazole, 1 mg mL^{-1} lysozyme, 1 mM β-ME, and 1 mM PMSF) on ice. Cells were lysed by running through a pre-cooled French press at 25,000 psi at least three times. The cell debris was spun down by centrifugation at 15,000 rpm for 90 minutes at 4 °C and the supernatants were collected. After this point, PARP2 was purified using the same protocol as described above for human PARP1. The extinction coefficient of full-length human PARP2 with a C-terminal His_6-tag is 1.05.

Bacterial expression and purification of human PARP5a and PARP10

The expression and purification of the catalytic domains of human PARP5a and human PARP10 were performed as previously described with minor modifications.[3,5] Sequence-verified plasmids encoding the catalytic domains of human PARP5a and PARP10 were transformed into

BL21 (DE3) *E. coli* for bacterial expression and purification. As in the procedures for PARP1 and PARP2, 5 mL of sterilized LB broth containing 50 μg/mL kanamycin was inoculated with the transformed BL21 (DE3) cells and cultured overnight at 37 °C at 220 rpm. The overnight cultures were then transferred into 1 L sterilized LB broth containing 50 μg/mL kanamycin and grown to OD_{600} = 1.2 – 1.4 at 37°C at 220 rpm. Expression of PARP5a and PARP10 were induced using a 500 mM stock of IPTG to a final concentration of 500 μM and grown overnight at 16 °C at 220 rpm. Cells were harvested by centrifugation at 4,000 × *g* for 30 minutes at 4 °C, and cell pellets were resuspended in 25 mL cell lysis buffer (20 mM Tris pH 7.5, 500 mM NaCl, 20 mM imidazole, 1 mg mL^{-1} lysozyme, 1 mM β-ME, and 1 mM PMSF). In the case of PARP10, β-ME was replaced with 1 mM tris(2-carboxyethyl)phosphine (TCEP) in the cell lysis buffer. Cells were lysed by running through a pre-cooled French press at 25,000 psi three times. The cell debris was spun down by centrifugation at 15,000 rpm for 50 minutes at 4 °C and the supernatants were collected. The collected supernatants were then filtered through 0.45 μm membranes under vacuum and subsequently loaded onto the nickel column and eluted from the nickel column as described above. β-ME or TCEP was added to the eluted proteins to a final concentration of 1 mM for PARP5a or PARP10, respectively, before concentrating to 1 mL with a 10 kDa MWCO ultra-15 centrifugal filter unit. The concentrated proteins were then further purified through an ÄKTA pure FPLC with a Superdex 75 Increase 10/300 column pre-equilibrated with gel-filtration buffer (20 mM Tris pH 7.5, 300 mM NaCl, 10% glycerol, and 1 mM DTT). An isocratic elution method with a flow rate of 0.5 mL min^{-1} was used to collect 0.5 mL fractions, which were subsequently examined by SDS-PAGE gels to identify fractions containing the desired proteins. Fractions containing pure PARP5a or PARP10 were combined and concentrated with 10 kDa centrifugal filter units. Protein

concentrations were determined by UV absorbance at 280 nm using extinction coefficients of 0.753 and 0.762 for PARP5a and PARP10, respectively.

Substrate activities of NAD^+ or NAD^+ analogues for PARPs

Purified human PARP1 (3 μM) and human PARP2 (6 μM) were pre-activated in a solution of 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 100 mM NaCl, and 1 mM DTT containing 0.1 mg mL^{-1} activated DNA (Sigma Aldrich, D4522) with or without olaparib (100 μM) for 30 minutes at 30 °C before adding 300 μM NAD^+ or NAD^+ analogues. After the addition of NAD^+ or NAD^+ analogues, auto-modification reactions of PARP1 and PARP2 were incubated for 2 hours at 30 °C. Conversely, purified catalytic domains of PARP5a and PARP10 (20 μM) were immediately incubated with 300 μM NAD^+ analogues in a solution of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, and 1 mM DTT with or without olaparib (100 μM) for 2 hours at room temperature. All reactions were quenched by adding 100 μM olaparib after the indicated incubation time. For detection of biotinylated PARPs, reaction mixtures were further labeled with alkyne-biotin (for ADO-4′-N_3-NAD^+ and NR-3′-N_3-NAD^+) or azide-biotin (for 2-a-NAD^+ or 6-a-NAD^+) using copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC). Click reactions were performed for 1.5 hours at room temperature using 250 μM $CuSO_4$, 500 μM tris-hydroxypropyltriazolylmethylamine (THPTA), 2.5 mM sodium ascorbate, and 100 μM biotin-PEG4-alkyne or biotin-PEG4-azide.

Automodification levels of ADP-ribosylated PARP1 and PARP2 were evaluated by immunoblots using an anti-PAR monoclonal antibody (clone: 10H, Santa Cruz Biotechnology sc-56198) to detect the formation of poly-ADP-ribose polymers. NuPAGE LDS sample buffer (Thermo Fisher, NP0007) was added to unlabeled, quenched reaction mixtures, which were then boiled at 95°C for 5 minutes and run on precast SDS-PAGE gels. After transferring onto PVDF

membranes, proteins were blocked with 5% milk in PBS with 0.05% tween-20 (PBS-T) for 2 hours and washed with PBS-T, at which point the anti-PAR antibody was applied at a 1:500 ratio in PBS-T overnight at 4 °C. After washing three times with PBS-T, a goat anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP) (Thermo Fisher, G-21040) was applied as the secondary antibody at a ratio of 1:1000 in PBS-T for 2 hours at room temperature. After a second round of washing, SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher, 34580) was applied, and the blots were imaged using a ChemiDoc Touch Gel Imaging System.

Automodification levels of biotinylated PARPs were evaluated directly by immunoblots using a streptavidin-HRP conjugate (R&D Systems: DY998; 1:200 dilution). NuPAGE LDS sample buffer (Thermo Fisher, NP0007) was added to biotin-labeled reaction mixtures, run on precast SDS-PAGE gels, and transferred onto PVDF membranes. The blocking reagent was substituted for 3% bovine serum albumin (BSA) in PBS-T. Loading controls for PARP1, PARP5a, and PARP10 were performed using an anti-His_6 antibody (clone: HIS.H8, Thermo Fisher MA1-21315; 1:2000 dilution) as the primary antibody, while loading controls for PARP2 were performed using an anti-PARP2 antibody (clone: F-3, Santa Cruz Biotechnology sc-393310; 1:500 dilution) as the primary antibody. As described above, a goat anti-mouse IgG antibody conjugated to HRP was used as the secondary antibody to generate chemiluminescent signal for the loading controls.

Kinetic analysis of PARP1 with NR-3′-N_3-NAD^+ and ADO-4′-N_3-NAD^+

Kinetic analysis of PARP1 with NR-3′-N_3-NAD^+ and ADO-4′-N_3-NAD^+ was performed as previously described with minor modifications.[3–5] Automodification of PARP1 was carried out in 50 μL reactions. Purified human PARP1 (1 μM) was pre-activated in a solution of 50 mM Tris-

HCl, pH 7.5, 20 mM $MgCl_2$, 100 mM NaCl, and 1 mM DTT containing 0.1 mg mL^{-1} activated DNA (Sigma Aldrich, D4522) for 30 minutes at 30 °C before adding NAD^+ analogues. NR-3′-N_3-NAD^+ was added to final concentrations of 50, 100, 400, and 600 μM and incubated at 30 °C for 0, 10, 20, or 30 minutes. ADO-4′-N_3-NAD^+ was added to final concentrations of 200, 400, 800, and 1000 μM and incubated at 30°C for 0 or 120 minutes. Reactions were quenched at each of the indicated time points with additions of 50 μL ice-cold 20% trichloroacetic acid (w/v).

Reaction samples were analyzed by a Waters HPLC using a Luna C18 column (5 μm, 100 Å, 150 × 4.6 mm) (Phenomenex Inc., 00F-4252-E0) with a mobile phase A: 0.1% formic acid (aq.) and mobile phase B: 0.1% formic acid in acetonitrile and detection at UV absorbance at 260 nm. The gradient program was set up as follows: 1 mL min^{-1} at a gradient of 0-2 min 5% B, 2-4 min 5-10% B, 4-6 min 10-20% B, 6-12 min 20-50% B, 12-17 min 50-100%B, and 17-20 min 100-5% B.

Substrate activity of ADO-4′-N_3-NAD^+ with HEK293T cell lysates

HEK293T cells were grown to 70% confluency in DMEM with 10% FBS in T25 flasks prior to harvesting with trypsin. The cell pellet was resuspended in 200 μL cell lysis buffer (25 mM Tris-HCl pH 7.5, 50 mM NaCl, 10% glycerol, 0.2% Triton X-100, and halt protease inhibitor cocktail (Thermo Fisher Scientific, 78430)) and placed on ice for 1 hour. Cellular debris was spun down at 14,000 × *g* at 4°C for 15 minutes. The supernatant was collected and protein concentrations of cell lysate were determined by Bradford assays.

To evaluate the substrate activity of ADO-4′-N_3-NAD^+ in HEK293T cell lysates, 10 μg of cell lysates were incubated with 600 μM NAD^+ or ADO-4′-N_3-NAD^+ in PBS with 100 ng μL^{-1} activated DNA (Sigma Aldrich, D4522) and 10 μM of poly(ADP-ribose) gylcohydrolase (PARG)

inhibitor PDD00017273 (Sigma Aldrich) overnight at room temperature. Olaparib (100 μM) was included as a negative control. After overnight incubation, reaction mixtures were split evenly for immunoblot analysis by AF1521 macrodomain (Sigma Aldrich, MABE1016; 1:1000 dilution), the streptavidin-HRP conjugate following CuAAC to label ADP-ribosylated proteins, and a GAPDH loading control antibody (Thermo Fisher Scientific, MA5-15738).

Recognition of auto-PARylated PARP1 by reader proteins

Master auto-modification reactions (50 μL reaction volume) of purified, pre-activated human PARP1 (3 μM) with 400 μM NAD^+ or NAD^+ analogues were carried out in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 100 mM NaCl, and 1 mM DTT containing 0.1 mg mL^{-1} activated DNA at 30 °C overnight. After overnight incubation, the master reaction mixtures were split into 10 μL aliquots. To verify automodification levels, one aliquot was used for CuAAC and subsequent detection by the streptavidin-HRP conjugate. The remaining aliquots were used for immunoblot analysis using an anti-PAR antibody (clone: 10H, Santa Cruz Biotechnology sc-56198; 1:500 dilution), AF1521 macrodomain (Sigma Aldrich MABE1016; 1:1000 dilution), a RNF146 WWE domain (Sigma Aldrich MABE1031; 1:1000 dilution), and an anti-His_6 antibody (clone: HIS.H8, Thermo Fisher MA1-21315; 1:2000 dilution). Briefly, reaction aliquots were prepared with NuPAGE LDS sample buffer, boiled, and run on precast SDS-PAGE gels. After transferring onto PVDF membranes, the proteins were blocked with 5% milk in PBS-T and washed before applying the indicated primary detection agents for 2 hours at room temperature. After washing three times with PBS-T, a goat anti-mouse IgG antibody-HRP conjugate (Thermo Fisher, G-21040; 1:1000 dilution) or a goat anti-rabbit IgG antibody-HRP conjugate (Thermo Fisher, G-21234; 1:1000 dilution) was applied for 2 hours at room temperature for secondary antibody detection of anti-PAR and anti-His_6 antibody binding or macrodomain and WWE domain binding,

respectively. After washing, SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher, 34580) was applied, and the blots were imaged using the ChemiDoc Touch Gel Imaging System.

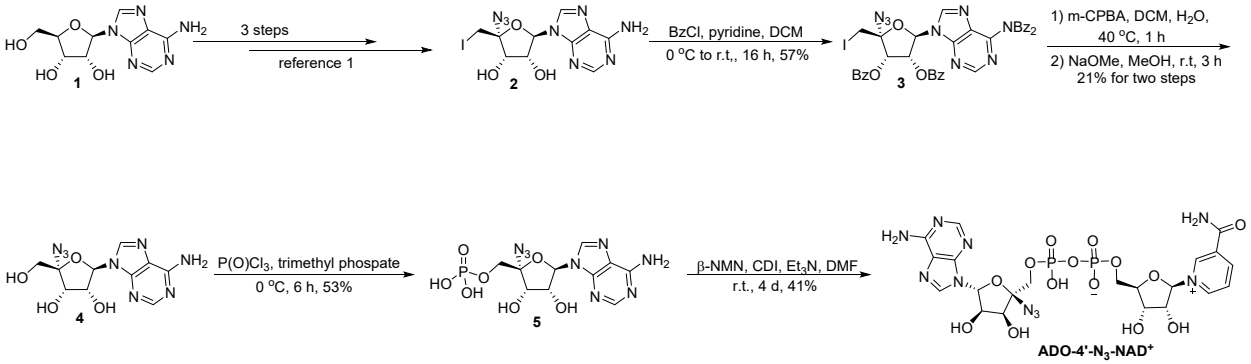
Recognition of auto-MARylated PARP10 by reader proteins

Master auto-modification reactions (40 μL reaction volume) of the purified catalytic domain of human PARP10 (20 μM) with 400 μM NAD^+ or ADO-4′-N_3-NAD^+ were carried out in a solution of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, and 1 mM DTT with or without olaparib (100 μM) overnight at room temperature. After overnight incubation, the master reactions were split into 10 μL aliquots, which were used for immunoblot analysis by AF1521 macrodomain (Sigma Aldrich, MABE1016; 1:1000 dilution), PARP14 macrodomain (Sigma Aldrich MABE1076; 1:1000 dilution), and an anti-His_6 antibody (clone: HIS.H8, Thermo Fisher MA1-21315; 1:2000 dilution). As described above, a goat anti-rabbit IgG antibody-HRP conjugate (Thermo Fisher, G-21234; 1:1000 dilution) or a goat anti-mouse IgG antibody-HRP conjugate (Thermo Fisher, G-21040; 1:1000 dilution) was used as the secondary antibody to detect macrodomain binding or anti-His_6 antibody binding, respectively. Blots were imaged using the ChemiDoc Touch Gel Imaging System.

Statistical analysis

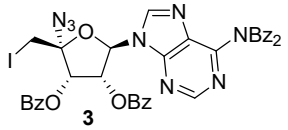
One-tailed unpaired *t* tests were performed for densitometric analyses of protein ADP-ribosylation revealed by immunoblots. $p < 0.05$ was used as a threshold for statistical significance. Significance levels were defined as: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Data are shown as mean ± standard deviation (n = 2 or 3). Statistical analyses were performed using GraphPad Prism (GraphPad Software).

Chemical synthesis and characterization of ADO-4′-N_3-NAD^+.



Scheme 1. Chemical synthesis of ADO-4′-N_3-NAD^+.

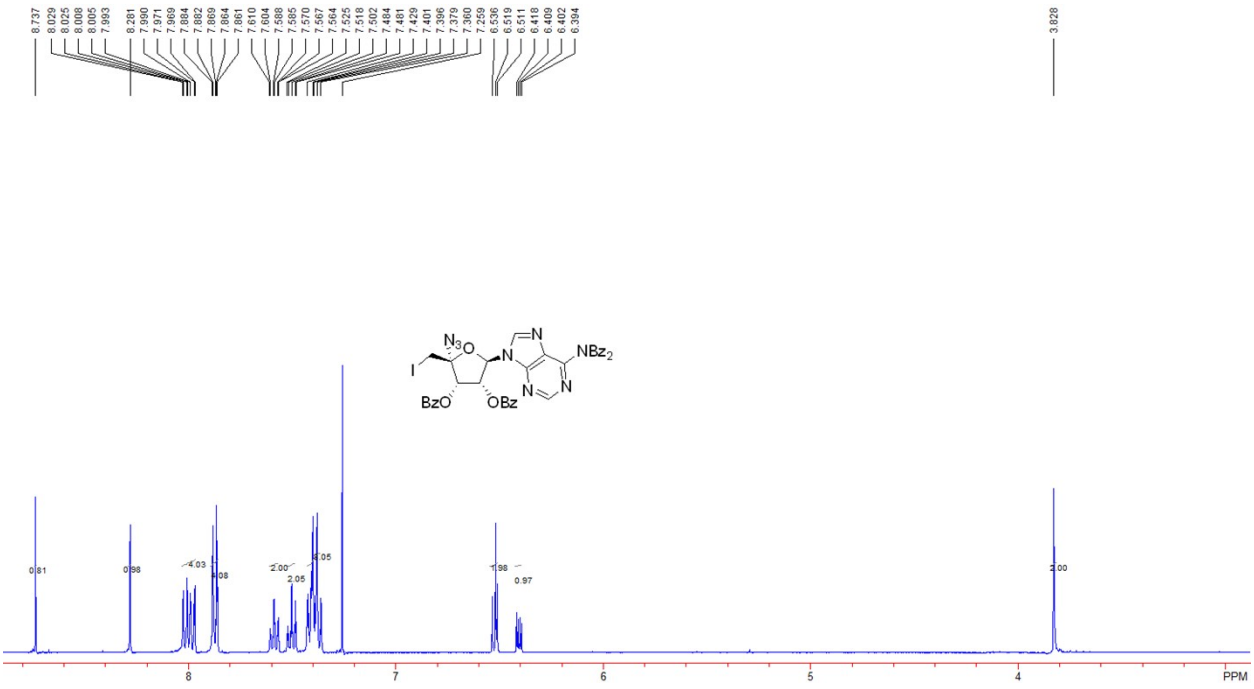
General materials and methods for compound synthesis. ^{1}H NMR spectra were recorded on an Oxford AM-400 spectrometer for solution in $CDCl_3$, CD_3OD or D_2O. Coupling constants *J* are shown in Hz. ^{13}C NMR spectra were recorded on an Oxford AM-400 spectrophotometer (100 MHz) with complete proton decoupling spectrophotometer (CD_3OD: 49.0 ppm). Flash column chromatography was performed using 230-400 mesh silica gel (Sigma-Aldrich, St. Louis, MO). For thin-layer chromatography (TLC), silica gel plates (Sigma-Aldrich: GF254) were used. HPLC was performed on a Waters 2487 series with a C18 Kinetex column (5 μm, 100 Å, 150×10.0 mm, Phenomenex Inc, Torrance, CA). All other reagents were purchased from readily available commercial sources and used without further purification.

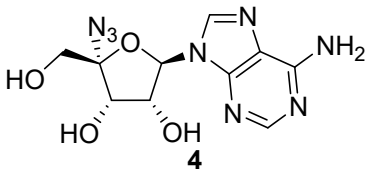


*General procedure for the synthesis of compound **3***: To a solution of compound **2** (1.25 g, 3.0 mmol) in a mixture of anhydrous DCM (20 mL) and anhydrous pyridine (20 mL) was added BzCl (2.1 mL, 18.0 mmol, 6 eq) at 0 °C. Then the reaction mixture was allowed to warm to room temperature. After stirring for 24 hours, the reaction was quenched with MeOH (10 mL) and the mixture was concentrated under reduced pressure to give a residue. The residue was dissolved in EtOAc (100 mL), and the organic phase was washed successively with saturated aqueous $CuSO_4$

(5×50 mL), brine (50 mL), dried over anhydrous Na_2SO_4, filtered, concentrated, and purified by a flash column chromatography on silica gel to afford the compound **3** (1.4 g, 57%).

(2S,3S,4R,5R)-2-azido-5-(6-(N-benzoylbenzamido)-9H-purin-9-yl)-2-(iodomethyl)tetrahydrofuran-3,4-diyl dibenzoate (3). This is a known compound.[9] ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.83 (s, 2H, CH_2), 6.40 (dd, 1H, J = 6.4, 3.2 Hz, CH), 6.51-6.54 (m, 2H, 2CH), 7.36-7.42 (m, 8H, ArH), 7.48-7.53 (m, 2H, ArH), 7.56-7.61 (m, 2H, ArH), 7.86-7.88 (m, 4H, ArH), 7.97-8.03 (m, 4H, ArH), 8.28 (s, 1H, ArH), 8.74 (s, 1H, ArH).





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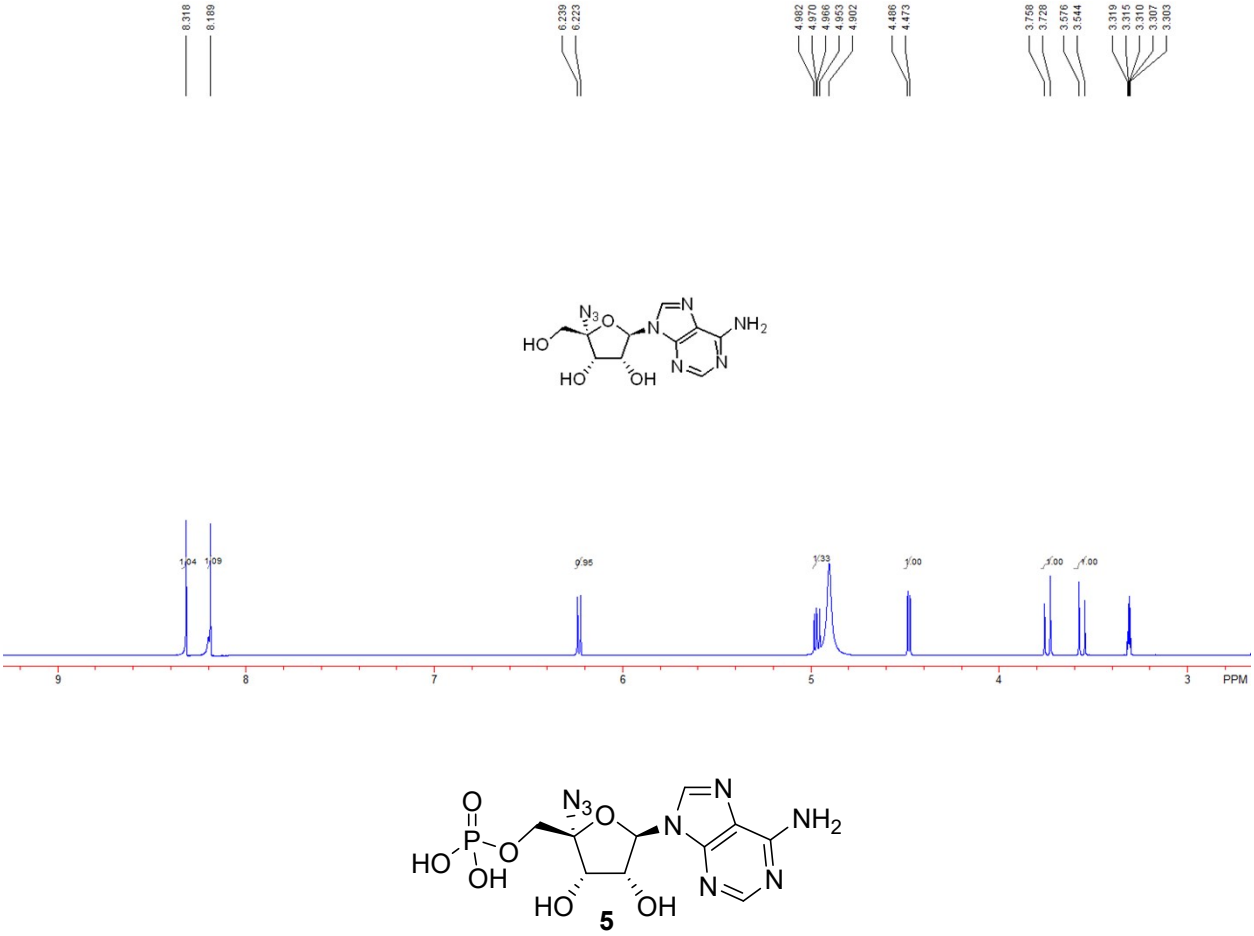
*General procedure for the synthesis of compound **4***: To a solution of compound **3** (1.0 g, 1.2 mmol) in CH_2Cl_2 (30 mL, saturated with 1% of water) was added 75% m-CPBA (*m*-chloroperbenzoic acid) (1.66 g, 7.2 mmol, 6 eq), and the reaction was stirred at 40 °C for 1 h. EtOAc (80 mL) was added and then washed with a saturated solution of $Na_sS_2O_3$ (3×50 mL). The organic layer was dried using $MgSO_4$, and then the solvent was removed under reduced pressure. The yellow solid was dissolved in a solution 1 N MeONa in MeOH (6 mL) for 1 h. Then the mixture was

concentrated under reduced pressure to give a residue, which was purified by a flash column chromatography on silica gel to afford the compound **4** (77 mg, yield: 21%).

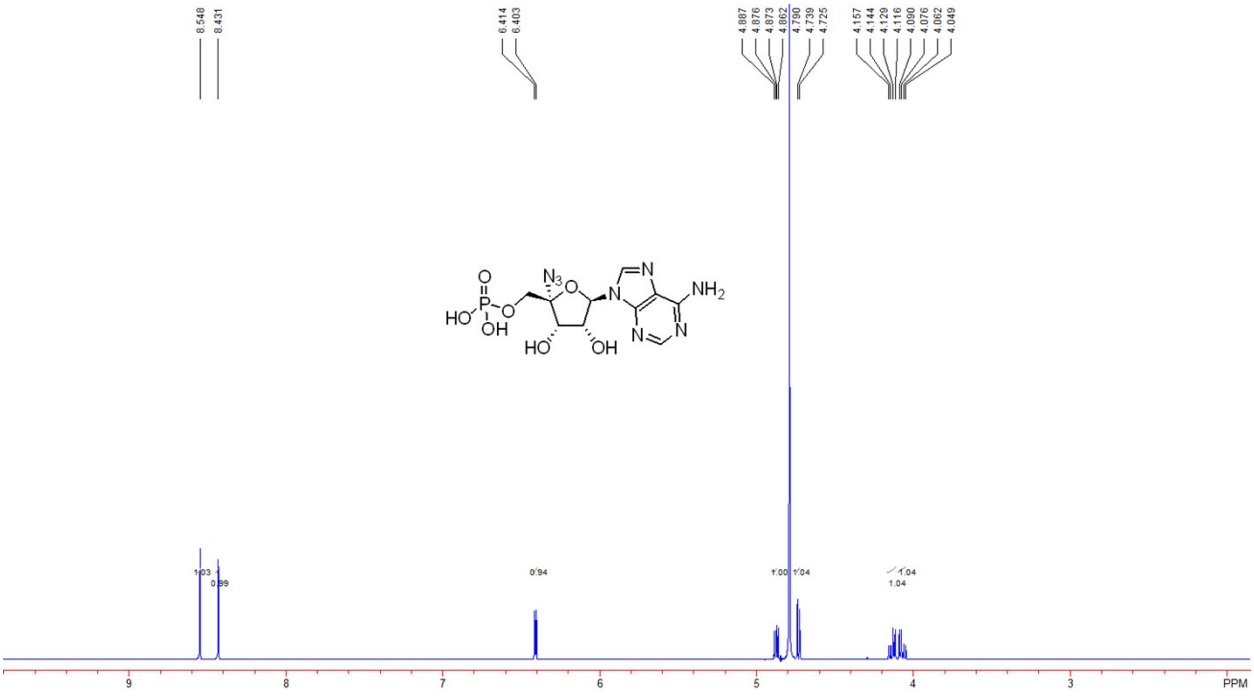
(2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-2-azido-2-(hydroxymethyl)tetrahydrofuran-3,4-diol (4). This is a known compound.[1] ^{1}H NMR (400 MHz, CD_3OD): δ 3.56 (d, 1H, J = 12.4 Hz, CH_2), 3.74 (d, 1H, J = 12.4 Hz, CH_2), 4.48 (d, 1H, J = 5.2 Hz, CH), 4.97 (dd, 1H, J = 6.4, 5.2 Hz, CH), 6.23 (d, 1H, J = 6.4 Hz, CH), 8.19 (s, 1H, ArH), 8.32 (s, 1H, ArH).



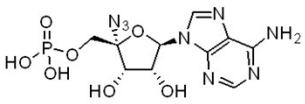
*General procedure for the synthesis of compound **5***: To a stirred solution of compound **4** (62 mg, 0.2 mmol) in trimethylphosphate (2 mL) was added $P(O)Cl_3$ (56 µL, 0.6 mmol, 3 eq) at 0 ˚C and the resulting mixture was stirred at 0 ˚C for 6 hours. A few drops of water were then added to quench the reaction. The reaction was then concentrated *in vacuo* and the crude product was purified via HPLC using the C18 Kinetex column (5 µm, 100 Å, 150×10.0 mm) (mobile phase A: 0.1% formic acid (aq), mobile B: 0.1% formic acid in acetonitrile; flow rate = 2.0 mL min^{-1}; 0-2 min: 0-4% B, 2-4 min: 4-10% B; 4-8 min: 10-20% B; 8-9 min: 20% B; 9-12 min: 20-50% B; 12-14

min: 50-0% B). Fractions containing the desired product were concentrated and lyophilized to yield the desired products **5** (41 mg, yield: 53%) as a colorless solid.

((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-2-azido-3,4-dihydroxytetrahydrofuran-2-yl)methyl dihydrogen phosphate (5). ^{1}H NMR (400 MHz, D_2O): δ 4.07 (dd, 1H, *J* = 11.2, 5.2 Hz, CH_2), 4.14 (dd, 1H, *J* = 11.2, 5.2 Hz, CH_2), 4.73 (d, 1H, *J* = 5.6 Hz, CH), 4.87 (dd, 1H, *J* = 5.6, 4.4 Hz, CH), 6.41 (d, 1H, *J* = 4.4 Hz, CH), 8.43 (s, 1H, ArH), 8.55 (s, 1H, ArH); ^{13}C NMR (100 MHz, D_2O): δ 65.7 (d, *J* = 3.8 Hz), 71.4, 73.3 (d, *J* = 3.3 Hz), 89.1 (d, *J* = 4.8 Hz), 98.3 (d, *J* = 9.9 Hz), 118.6, 142.5, 144.6, 148.2, 149.9; ^{31}P NMR (162 MHz, D_2O): δ -0.45; HRMS (ESI) for $C_{10}H_{14}N_8O_7P^+$ $(M+H)^+$: Calcd.: 389.0723 Da; Obs: 389.0729 Da.




149.868
148.221
144.606
142.545
118.639
98.301
98.202
89.148
89.100
73.357
73.324
71.378
65.741
65.703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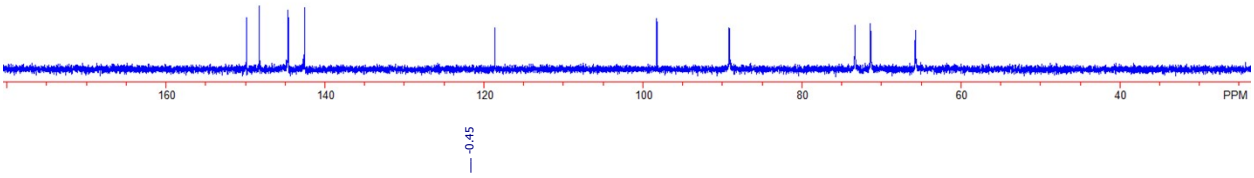
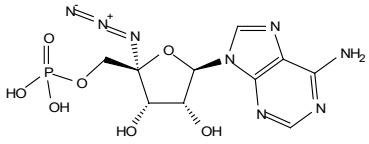
HO
P
O
OH
N3
O
N
N
NH2
N
N
HO
OH


160
140
120
100
80
60
40
PPM
-0.45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N+
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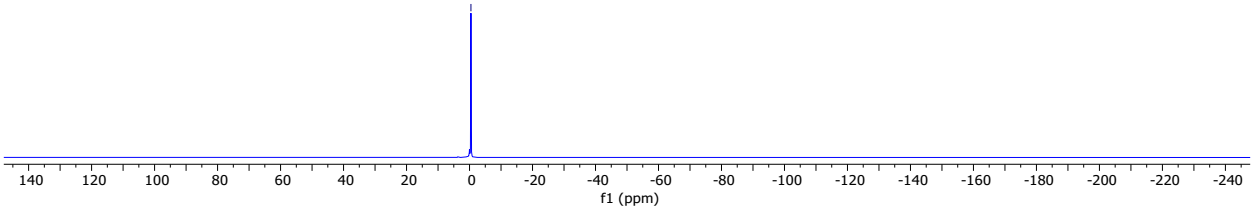
O
HO
P
O
OH
HO
OH
N
N
N
NH2
N


140
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
-140
-160
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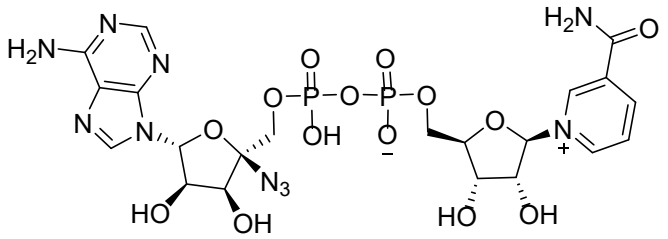
-200
-220
-240
f1 (ppm)

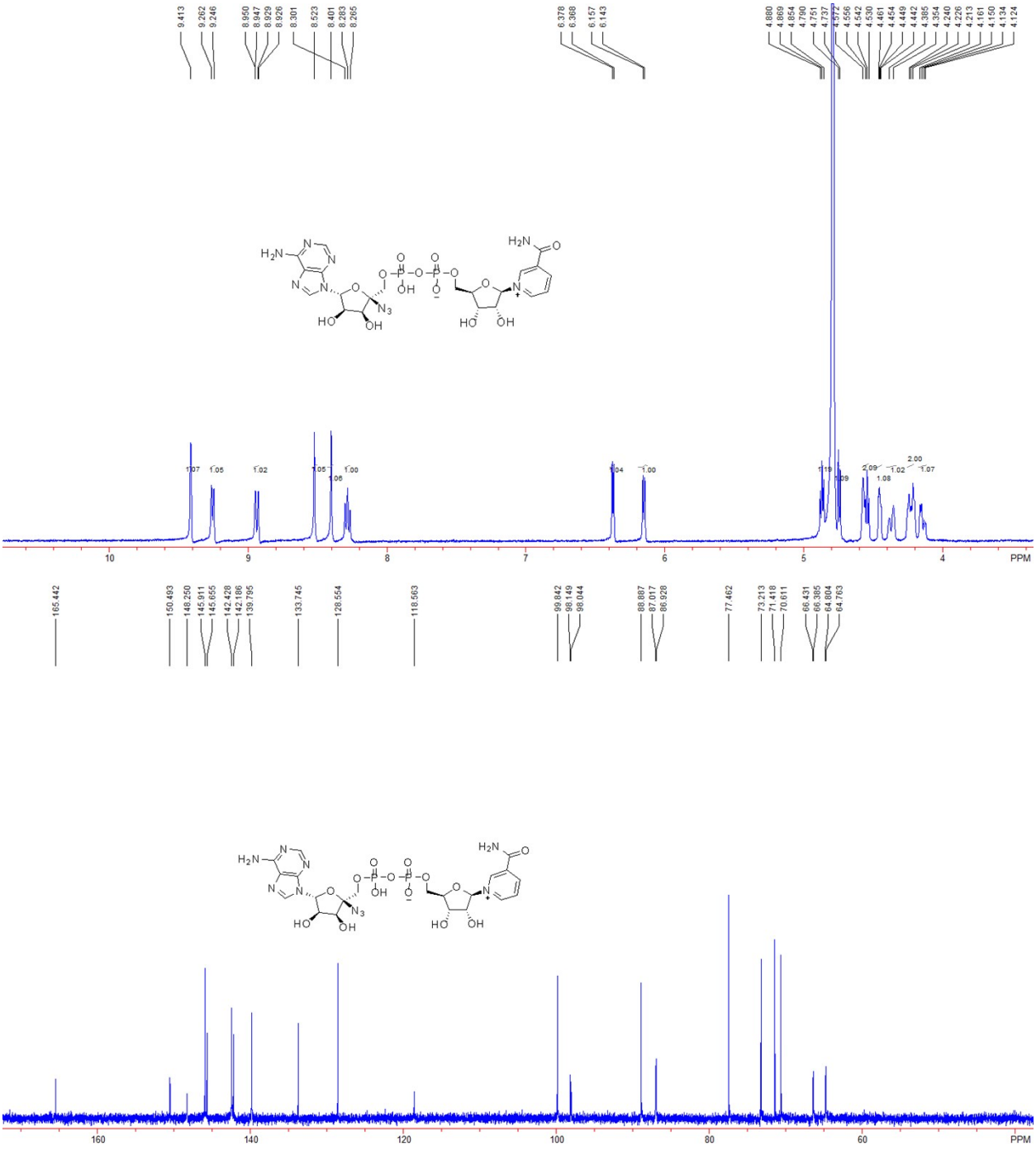


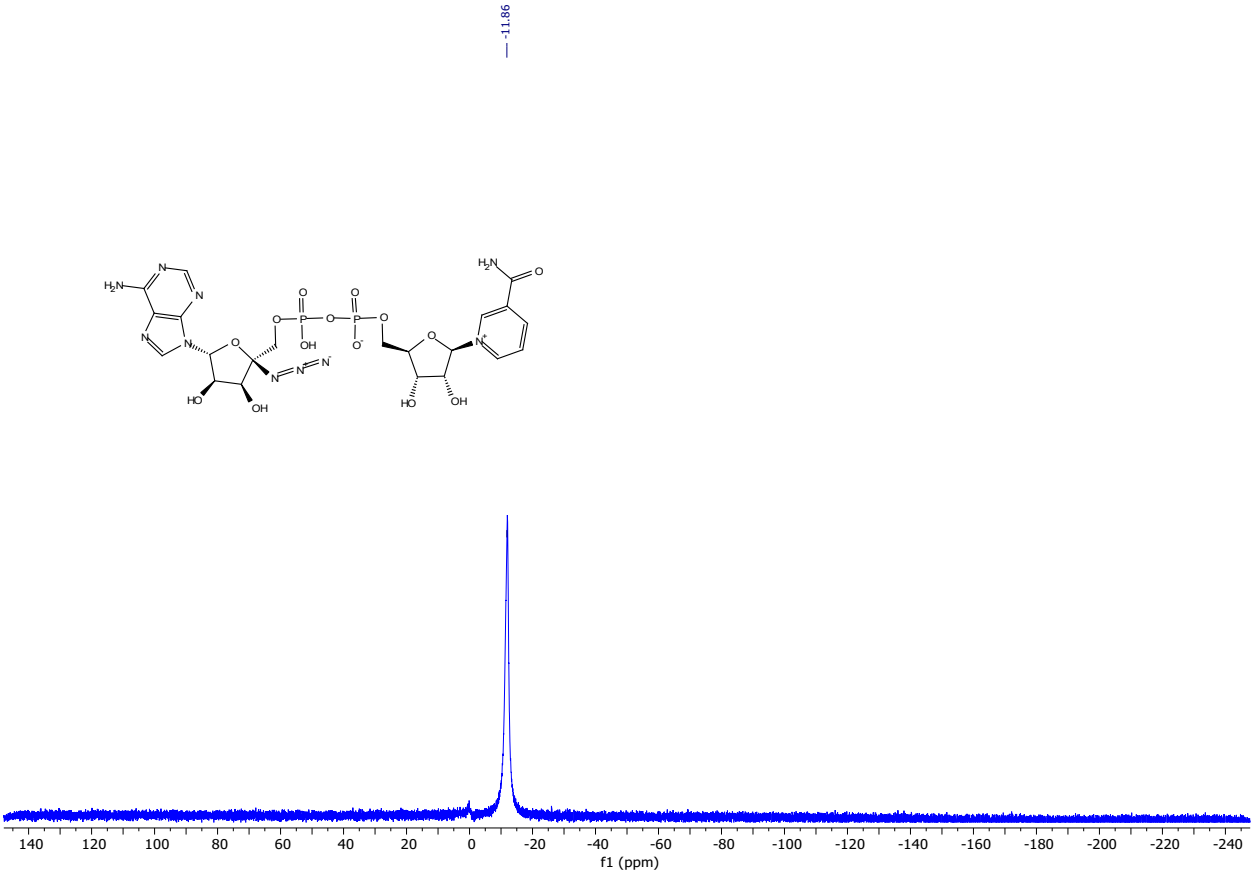
ADO-4'-N_3-NAD^+

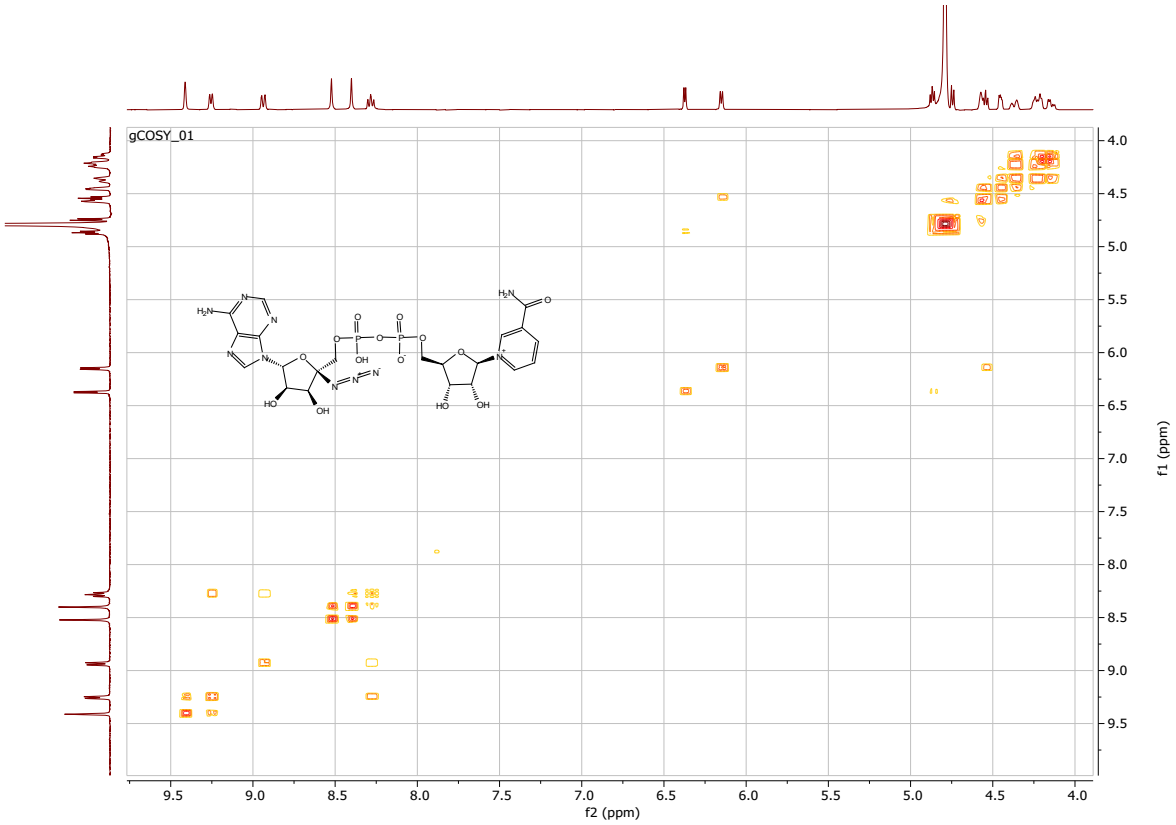
*General procedure for the synthesis of compound **ADO-4′-N_3-NAD^+***: To a stirred solution of **5** (39 mg, 0.1 mmol) in dried DMF (2 mL) were added 1,1-carbonyldiimidazole (CDI) (63 mg, 0.50 mmol, 5 eq) and triethylamine (23 μL, 0.16 mmol. 1.6 eq). The reaction mixture was stirred at room temperature for 10 hours, and then quenched with 0.1 mL dried methanol. The solvent was removed under vacuum and the residue was co-evaporated 3 times each with 1 mL of dried DMF. The activated 5′-AMP analogue was dissolved in dried DMF (1 mL) and β-NMN (50 mg, 0.15 mmol, 1.5 eq) was added. After stirring at room temperature for 4 days, H_2O (20 mL) was added to quench the reaction at 0 ˚C. The resulting mixture was continued stirring at room temperature for 48 hours. The reaction was then concentrated *in vacuo* and the crude product was purified via HPLC using the C18 Kinetex column (5 μm, 100 Å, 150×10.0 mm) (mobile phase A: 0.1% formic acid (aq), mobile B: 0.1% formic acid in acetonitrile; flow rate = 2.0 mL min^{-1}; 0-2 min: 0-4% B, 2-4 min: 4-10% B, 4-6 min: 10-20% B, 6-12 min: 20-50% B, 12-17 min: 50-100% B, 17-20 min: 100-0% B) with detection of UV absorbance at 260 nm. Fractions containing the desired product were concentrated and lyophilized to yield the **ADO-4′-N_3-NAD^+** (29 mg, yield: 41%) as a colorless solid.

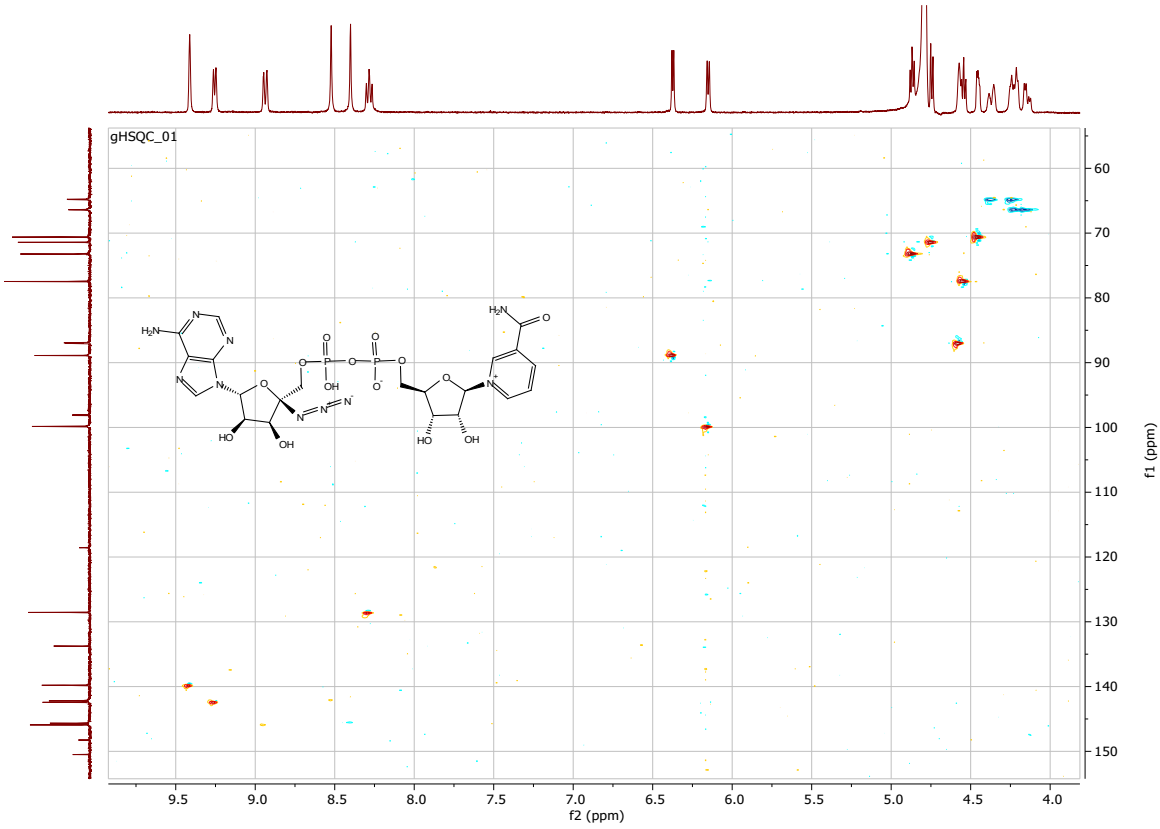
1-((2R,3R,4S,5R)-5-(((((((((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-2-azido-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryl)oxy)oxidophosphoryl)oxy)methyl)-3,4-dihydroxytetrahydrofuran-2-yl)-3-carbamoylpyridin-1-ium (ADO-4′-N_3-NAD^+). ^{1}H NMR (400 MHz, D_2O): δ 4.12-4.16 (m, 1H, CH_2), 4.21-4.24 (m, 2H, CH_2), 4.35-4.39 (m, 1H, CH_2), 4.44-4.46 (m, 1H, CH), 4.53-4.57 (m, 2H, 2CH), 4.74 (d, 1H, *J* = 5.6 Hz, CH), 4.85-4.88 (m, 1H, CH), 6.15 (d, 1H, *J* = 5.6 Hz, CH), 6.37 (d, 1H, *J* = 4.0 Hz, CH), 8.28 (t, 1H, *J* = 7.2 Hz, ArH), 8.40 (s, 1H, ArH), 8.52 (s, 1H, ArH), 8.94 (dd, 1H, *J* = 8.4, 1.2 Hz, ArH), 9.25 (d, 1H, *J* = 6.4 Hz, ArH), 9.41 (s, 1H, ArH); ^{13}C NMR (100 MHz, D_2O): δ 64.7-64.8 (m), 66.38-66.43 (m), 70.6, 71.4, 73.2, 77.5, 87.0 (d, *J* = 18.9 Hz), 88.9, 98.1 (d, *J* = 10.5 Hz), 99.8, 118.6, 128.6, 133.7, 139.8,

142.2, 142.4, 145.7, 145.9, 148.3, 150.5, 165.4; ^{31}P NMR (162 MHz, D_2O): δ -11.86 (br); HRMS (ESI) for $C_{21}H_{26}N_{10}O_{14}P_2Na^+$ $(M+Na)^+$: Calcd.: 727.1003 Da; Obs: 727.1002 Da.




-11.86
140
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
-140
-160
-180
-200
-220
-240
f1 (ppm)


gCOSY_01
f2 (ppm)
f1 (ppm)


gHSQC_01
f2 (ppm)
f1 (ppm)

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