ENZYMATIC SYNTHESIS OF DEFINED-LENGTH POLY(ADP-RIBOSE) 
AND 
THE INVESTIGATION OF CELL-PERMEABLE POLY(ADP-RIBOSE) 
GLYCOHYDROLASE INHIBITORS 

BY 
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ABSTRACT

Poly(ADP-ribosylation) (PARylation) is an important post-translational modification that maintains genomic stability in a cell. Engaging in important cellular processes such as DNA repair and cell death signaling, PARylation has gathered considerable interest as a target for genotoxic chemotherapy against cancer cells. To this end, various Poly(ADP-ribose) Polymerase (PARP) inhibitors have been developed to induce sensitivity to genotoxic stress in BRCA-mutated cancer cells, and Poly(ADP-ribose) Glycohydrolase (PARG) inhibition is investigated as an alternate pathway to PARP inhibition in genotoxic chemotherapy. However, little is known about the exact mode of interaction between PAR and different proteins mainly due to the fact that PARP produces polydisperse mixtures of PAR through a heterogeneous modification process. To tackle these problems, a controlled enzymatic synthesis pathway of PAR has been investigated through the use of masked β-NAD⁺ derivatives that can homogenously and monomerically modify PARP. In order to verify its ability to modify PARP, a sample of proparagyl-β-NAD⁺ derivative was used in an automodification assay with hTNKS-1. In addition, a PARG inhibitor prodrug in the form of an alanine-ester-masked ADP-HPM was developed as a cell-permeable PARG inhibitor to investigate its effect in a whole cell. In order to verify its activity, an in vitro experiment of the enzymatic cleavage of its masking group with HINT-1 was performed and the results were analyzed via LC/MS.
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CHAPTER 1
PAR-CYCLE AS TARGET FOR CANCER TREATMENT

1.1. Introduction: The PAR-cycle

Poly(ADP-Ribosylation) (PARylation) is an important post-translational modification that governs important cellular processes, ranging from DNA repair, transcription, and telomere maintenance to cell division and cell death.\textsuperscript{1-4} Figures 1.1 and 1.2 illustrate the outline of the general process: When DNA damage is detected, Poly(ADP-Ribose) Polymerase (PARP) binds to the damage site to release chemical signals in the form of PARylation, where $\beta$-NAD$^+$ is consumed to polymerize into Poly(ADP-Ribose) (PAR). These polymers act as molecular scaffolds for recruitment of DNA repairing factors such as XRCC-1 to the DNA damage site. Once DNA damage is repaired, Poly(ADP-Ribose) Glycohydrolase (PARG) cleaves PAR from the inactivated PARP complex and further degrades PAR down to monomeric ADP-Ribose units.

![Figure 1.1. PARylation cycle with minor DNA damage.](image)

In case the extent of damage is minor, PARP produces PAR from $\beta$-NAD$^+$ to recruit DNA repair factors.
Figure 1.2. PARylation cycle with severe DNA damage. When the DNA damage is severe, PAR is overproduced and cleaved by PARG. The resulting long-chain PAR interacts with the cell’s mitochondria to trigger PAR-mediated cell death known as PARthanatos.

On the other hand, if the DNA damage is severe and beyond repair, PARP overproduces PAR, resulting in longer chains of PAR that act as cell death signals by interacting with the Apoptosis Inducing Factor (AIF) of the cell’s mitochondria; thus, triggering a sequence of cell death known as PARthanatos. Due to its ability to induce cell death under increased genotoxic stress, manipulation of the PAR-cycle has been investigated as a means to supplement genotoxic chemotherapy for cancer.

1.2. PARP-inhibition in cancer treatment

Various PARP inhibitors have been developed to treat patients with BRCA mutations (Figure 1.3). In normal cells, PARP-1 is recruited to the DNA damage site to perform Base-Excision Repair (BER) on Single-Strand Breaks (SSBs). In PARP-inhibited cells, the inability to
perform BER results in the conversion of SSBs to Double-Strand Breaks (DSBs). To repair DSBs, PARP1 binds to the damage site to recruit proteins MRE11 and NBS1 to carry out the error-free repair mechanism known as Homologous Recombination (HR)\(^7,8\) and PARylate a key protein, BRCA1, that contributes to HR-mediated DSB repairs.\(^9\) Therefore, in BRCA1/2 mutated patients, PARP inhibition can prevent HR-activation and force cells to follow an error-prone repair pathway known as Non-Homologous End Joining (NHEJ), which results in increased genotoxic sensitivity and the eventual cell death. Initial studies have shown promising results for PARP inhibitors in treatment of ovarian cancer and, as of 2015, PARP inhibitor Olaparib,
distributed by AstraZeneca, has been approved by the US Food and Drug Administration as a fourth-line monotherapy for ovarian cancer in BRCA1/2 mutated patients.\textsuperscript{6}

1.3. Investigations in PAR and PARG inhibition

Despite these promising results several questions remain in the field of PAR research and its application to cancer treatment: How does PAR interact with PAR-binding proteins? What is the chemical nature of the bond between PAR and its modification sites? How many PAR modification sites exist on PARP? To explore these questions, there is a need to develop a means to produce defined-length PAR as well as a controlled method of PAR-modification on PARP.

Additionally, are there other means to manipulate the PARylation cycle to induce cell death via PARthanatos? Given that severe DNA damage results in overproduction of long-chain PAR which can act as a death signal,\textsuperscript{10} inhibiting PARG may delay the degradation of long-chain PAR to small-chain PAR or ADP-ribose monomers as well as contribute to the inactivation of PARP by saturating its modification sites with PAR. To this end, investigations have been carried out on the possibilities of PARG inhibition as an alternative to PARP inhibition.

1.3.1. Enzymatic and chemical synthesis of PAR

Upon detection of DNA damage, PARP binds to the DNA damage site via its zinc fingers and produces PAR of various lengths through a process known as automodification during which PARP enzymatically produces PAR on other PARP molecules of close proximity. Figure 1.4 shows the heterogeneous pattern of PAR production by PARP-1. While some members of the PARP family are known to mono-ADP-ribosylate proteins, the lengths of PAR produced by PARP can range anywhere from 2 units to 200 units and may have branching units.\textsuperscript{11} While polydisperse samples of enzymatically-synthesized PAR are commercially available, defined-
length samples of PAR are currently unavailable through such means.

In 2015, Lambrecht and co-workers succeeded in chemically synthesizing di-ADP-ribose, the first case of a successful chemical synthesis of defined-length PAR unit in literature.\textsuperscript{12} Starting with adenosine and ribose, through 30 steps Lambrecht and co-workers were able to synthesize di-ADP-ribose in an overall yield of 15\%. The same year, Kistemaker and co-workers reported in literature a method to synthesize defined-length PAR units via solid phase support.\textsuperscript{13} Though the number of steps required to produce di-ADP ribose was shorter, the overall yield of

Figure 1.4. Heterogeneous modification pattern of PARP. A) Automodification of PARP follows a heterogeneous pattern where each PAR unit can range anywhere from 2 to 200 ADP-ribose units.\textsuperscript{11} B) Automodification of PARP follows a heterogeneous pattern where each PAR unit can form branches of irregular patterns.
the process was less than 1%, and the final product had a methyl acetal protecting group that could not be removed. Since then, there have been no reported cases in the literature of successful chemical synthesis of PAR units of longer length (greater than 5 ADPr units).

As described previously, PAR plays an important role in various cellular processes, ranging from DNA damage repairs to triggering cell death known as PARthanatos. Despite its pivotal role in such processes, little is known about the interaction between PAR and various proteins. To begin with, the number of PAR modification sites on PARP and the nature of the chemical bond between these modification sites and PAR have long been debated.\textsuperscript{14-16} With the heterogeneous modification pattern of PAR on PARP, the exact number of modification sites on PARP as well as the chemical bond of the modification cannot be elucidated. Furthermore, there needs to be a means to access defined-length PAR of longer length. With the available chemical methods developed currently, producing dimers and short-length PAR may be plausible, but producing medium-to-long-length PAR is impractical due to the low overall yields of the synthesis and required number of steps to chemically synthesize PAR.

1.3.2. Development of PARG Inhibitors

As discussed previously, several PARP inhibitors have been developed and are currently being evaluated in clinical trials for cancer therapy. The inhibition of a related enzyme, PARG, has also been suggested\textsuperscript{17,18} to achieve similar effects albeit via a different mechanism (Figure 1.5). The strategy of inducing cell death through enhanced sensitivity to genotoxic stress via PARG inhibition has garnered considerable interest for several reasons: PARG activity plays a key role in the initiation of cell death and the enzyme itself is an attractive pharmacological target due to its low cellular abundance, which is approximately 2,000 molecules per cell.\textsuperscript{17}
However, investigations of PARG inhibition are largely thwarted by the lack of a potent, specific, and cell-permeable inhibitor.

Figure 1.5. PARylation cycle with severe DNA damage. When the DNA damage is severe, PAR is overproduced and cleaved by PARG. The resulting long-chain PAR interacts with the cell’s mitochondria to trigger PAR-mediated cell death known as PARthanatos.

In 1995, Jacobson and co-workers reported a potent PARG inhibitor known as ADP-HPD (1.1) with an IC₅₀ value of 0.12 μM. Its potency and relatively accessible synthetic route of 12 steps makes it an ideal benchmark of PARG inhibition and an attractive target of studying PARG inhibition. However, the pyrophosphate moiety and multiple polar functional groups render the molecule extremely hydrophilic and therefore not cell-permeable. Jacobson and co-workers have tested the inhibition of bovine PARG (bPARG) by modified ADP-HPD (1.2, 1.3). While both molecules exhibited inhibition of bPARG in vitro, no cell-permeability assay was carried out to show that 1.3 can permeate cells.
<table>
<thead>
<tr>
<th>Compound</th>
<th>PARG IC(_{50}) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP-HPD (1.1)</td>
<td>0.12(^{19})</td>
</tr>
<tr>
<td>ADP-HPM (1.2)</td>
<td>3.07 (bPARG)(^{20})</td>
</tr>
<tr>
<td>1.3</td>
<td>9.5 (bPARG)(^{20})</td>
</tr>
<tr>
<td>Mono-galloyl glucose (1.4)</td>
<td>0.95(^{21})</td>
</tr>
<tr>
<td>RBPI3 (1.5)</td>
<td>5.8(^{17})</td>
</tr>
<tr>
<td>Phenolic hydrazide hydrazones (1.6)</td>
<td>1.0(^{22})</td>
</tr>
</tbody>
</table>

**Table 1.1.** PARG inhibitors and their potency.
Ever since the discovery of ADP-HPD, several other PARG inhibitors have been developed, with their inhibition effects shown in table 1.1.\textsuperscript{17,19-22} While none of them show potency as strong as that of ADP-HPD, no studies have been carried out to determine the cell permeability of the reported PARG inhibitors. In order to establish the efficacy of PARG inhibition in causing programmed cell death, a cell-permeable small molecule inhibitor of PARG needs to be developed.

1.4. Summary and outlook

Manipulation of the PAR cycle through PARP/PARG inhibition has become an attractive target for cancer therapy. However, several important questions remain in the field that arise from the lack of appropriate chemical tools to investigate the chemical biology behind the PAR-cycle. The lack of means to homogenously modify PAR has led to the inability to investigate the chemical nature of the PAR-PARP modification site bond and the count of the modification sites. The lack of means to control the modification pattern of PAR results in the lack of accessibility to defined-length PAR for protein assays. With currently available chemical means, one can synthesize dimers and trimers of PAR, but synthesizing PAR molecules any longer than trimers requires a large number of synthetic steps and consequently suffers from low overall yields. There is a need to control the modification pattern of PAR to investigate means to synthesize defined-length PAR.

Additionally, the lack of cell-permeable PARG inhibitors thwarts investigation of PARG inhibition as an alternative to PARP inhibition in genotoxic chemotherapy. While several potent PARG inhibitors have been developed, they display lack of cell permeability or conclusive studies have not yet been carried out for their cell permeability. In order to demonstrate PARG
inhibition as a plausible supplementary method to genotoxic chemotheraphy, in vivo studies of PARG inhibition need to be carried out. Therefore, there is an urgent need to develop cell-permeable PARG inhibitors that can demonstrate as potent inhibition as ADP-HPD.

1.5. References

2015, 30, 68 – 79.
CHAPTER 2
SYNTHESIS OF DEFINED-LENGTH PAR

The enzymatic synthesis experiment of PAR by PARP has been planned out with Michael Lambrecht. Portions of the synthetic work of masked β-NAD⁺ derivatives and biological assay by human Tankyrase-1 were performed in collaboration with Michael Lambrecht.

2.1. Controlled enzymatic synthesis of PAR

It is suggested that PAR interacts with various proteins that play important roles in cellular processes such as DNA repair and cell death; however, little is known about the specifics of the interaction between PAR and relevant proteins. To begin with, it is uncertain how PAR modifies PARP and how many modification sites there are on PARP. Furthermore, relationships between length of PAR and interaction with various proteins haven’t been established. Most of these questions couldn’t be answered due to the heterogeneous automodification pattern of PAR on PARP. If there were a means to homogenously modify PARP with PAR, not only could we answer the questions of PARP modification but also produce defined-length PAR to carry out bioassays to establish relationships between size of the polymer and its activity. To this end, we suggest a controlled enzymatic synthesis experiment of PAR.

As discussed in chapter 1, chemical synthesis of defined-length PAR is an arduous and inefficient way to produce PAR of desired length due to the number of steps required (>30 steps) and low overall yield (<15%).⁴ On the other hand, it is impossible to produce defined-length PAR through enzymatic synthesis with the original β-NAD⁺ feedstock because use of β-NAD⁺ results in a heterogeneous modification pattern.⁵ Since PARylation proceeds on the 2’-hydroxyl group of adenosine on ADP-ribose, using a modified β-NAD⁺ derivative with the 2’-hydroxyl
group in question masked can stop the modification at a monomeric level and thus homogenously modify PARP. If the masking group can be removed easily (e.g. through photochemical cleavage), this monomeric modification can be utilized to synthesize defined-length PAR

\[
\begin{align*}
A) & \quad \beta-NAD \xrightarrow{\text{PARP digestion}} \quad \text{PAR} \\
\ &= \ \text{ADPr units} \quad \text{Masking group} \\
\ &= \ \text{PARP}
\end{align*}
\]

\[
\begin{align*}
B) & \quad \text{PG-NAD} \xrightarrow{\text{Deprotection}} \quad \text{PG-NAD} \\
\ &= \ \text{PARP digestion} \quad \text{PARP} \\
\ &= \ \text{Regular, defined length PAR}
\end{align*}
\]

**Figure 2.1. Controlled enzymatic synthesis of PAR.** A) Traditionally, PAR is obtained by feeding PARP with regular $\beta$-NAD$^+$ as feedstock; however, this produces a polydisperse mixture of PAR with varying lengths. B) In this experiment, A masked $\beta$-NAD$^+$ derivative can be used to end the modification at a monomeric level, PAR can modify PARP in a homogenous fashion. If the masking group can be removed photolytically, this method can be used to synthesize defined-length PAR in a controlled fashion.
Figure 2.1 illustrates the experiment’s general procedure. Traditional PAR modification experiments are carried out by feeding PARP with β-NAD⁺, which proceeds to heterogeneously modify PARP. Digestion of the PAR-PARP complex with hydroxylamine will cleave PAR off PARP’s modification sites to yield a polydisperse mixture of PAR. In our experiment, a modified β-NAD⁺ derivative will be used as feedstock instead in order to homogenously and monomerically modify PARP. The 2’-hydroxyl group of the β-NAD⁺ derivative will be masked by either a photocleavable masking group or a proparagyl group to prevent further modification. If the masking group is photocleavable, then in a stepwise fashion it can be removed to allow homogenous modification with the same masked β-NAD⁺ substrate. This can be used to produce defined-length PAR in a controlled fashion.

The masking group of choice was the o-nitrobenzyl group. Removal of the masking group can be carried out by irradiation of long-wave uv light (~ 300 nm) under conditions that do not damage the substrate. For the irremovable masking group, the proparagyl group was chosen to use the enzymatically produced PAR in fluorophore experiments. Ideally the photocleavable β-NAD⁺ substrate would be fed into PARP first to form the backbone of PAR, then, after several iterations of this procedure, the proparagylated β-NAD⁺ substrate can be fed as the last block to cap PARylation at a desired length. Hydroxylamine cleavage would yield a proparagylated PAR of uniform length to be used in fluorophore experiments.

Figure 2.2 shows target molecules of this project as well as the original β-NAD⁺ (2.1) for structural comparison, and scheme 2.1 illustrates the retrosynthetic analysis of each target molecule. Both target molecules 2.2 and 2.3 can be synthesized from their respective masked AMP (2.4 and 2.5) and nicotinamide mononucleotide (2.7). Both masked AMPs 2.4 and 2.5 can be synthesized through masking group coupling followed by phosphorylation of adenosine (2.6),
and phosphate 2.7 can be synthesized from tetraacetyl ribose (2.8). Both starting materials are commercially available in large quantities for cheap prices.

Figure 2.2. Structures of β-NAD⁺, 2'-(o-nitrobenzyl)-β-NAD⁺, and 2’-proparagyl-β-NAD⁺. Based on the structure of β-NAD⁺ (2.1), photocleavable masking groups such as o-nitrobenzyl groups or fluorophore-coupling handles such as proparagyl groups will be used to mask the 2’-hydroxyl group to produce 2’-(o-nitrobenzyl)-β-NAD⁺ (2.2) and 2’-proparagyl-β-NAD⁺ (2.3), respectively.

Scheme 2.1. Retrosynthetic analysis of masked-β-NAD⁺. The molecule can be synthesized from a pyrophosphate coupling of masked AMP (2.4 or 2.5) and nicotinamide mononucleotide (2.6). Each of these molecules can be synthesized from commercially available sources of adenosine (2.6) and tetraacetyl ribose (2.8).
2.1.1. Building block synthesis

Scheme 2.2 outlines the general progress of the experiment. The initial benzylation to produce 2.9 and proparagylation to form 2.11 were carried out using a modified procedure adopted by Chaulk and co-workers. Both the protection reaction and the phosphorylation were carried out successfully, and, following the phosphorylation, each phosphate was purified via C-18 chromatography. For the pyrophosphate coupling step, the imidazolide activating group was chosen due to its simple synthesis and wide usage in pyrophosphate coupling. Both 2.10 and 2.12 were synthesized in moderate quantities with synthetically useful overall yields (Scheme 2.2.A.).
On the other hand, the synthesis of phosphate 2.7 was challenging (scheme 2.2.B.). Protocols used for the synthesis of 2.7 were adopted from procedures used by Tanimori and co-workers and Kaminishi.\textsuperscript{5,6} Following the glycosylation of tetraacetate 2.8 with niacinamide to produce triacetate 2.13, quenching the reaction with methanol as performed by Tanimori and co-workers resulted in large amounts of impurities in the product as well as extensive decomposition if the product was left in solution for too long. Hence, the product 2.13 was purified via C-18 chromatography without quenching the reaction. Initially the deprotection of the triacetate protecting groups was done by ammonia in methanol at 0°C; however, this resulted in a mixture of partially deprotected products and desired product 2.14. To ensure full deprotection of triacetate 2.13, sodium methoxide, a stronger base, was used at -20°C. The new condition resulted in full deprotection of 2.13 as desired and produced 2.14 in quantitative yields.

The phosphorylation of 2.14 was done with phosphorous V oxychloride as usually done on nucleosides and nucleotides; however, its purification proved to be finicky and demanding, as it required multiple ion exchange columns and a large volume of water. There were few reports available in the literature with regards to alternate purification methods. The pure product was available only after running a C-18 column, followed by anion exchange columns in bicarbonate form and formate form, then a cation exchange column in proton form.

In the synthesis of pyrophosphate-based compounds, the pyrophosphate coupling step is of utmost importance as it is usually the final step to the synthesis, yet it is one of the most demanding reactions as it is required to be carried out in water-free conditions with polar solvents. Even a small amount of water can significantly lower the yield of the compound as the activated AMP-imidazolide would be hydrolyzed to produce AMP, which cannot couple with phosphate 2.7 without the activating group. To ensure that the solvent was kept dry, for the
pyrophosphate coupling of 2.10 and 2.12 with 2.7, a small amount of magnesium sulfate was added to the reaction along with manganese II chloride, the lewis acid catalyst for the coupling (Scheme 2.3). Both pyrophosphates 2.2 and 2.3 were produced in moderate yields and purified via preparatory HPLC.

Scheme 2.3. Pyrophosphate coupling of 2.10 and 2.12. The coupling was carried out with anhydrous manganese II chloride in dry formamide. Magnesium sulfate was added as a dehydrating agent.

2.1.2. Automodification experiment with hTNKS-1

PARP-5, otherwise known as Tankyrase-1 (TNKS-1) is one of the enzymes that constitutes the PARP family, a group of enzymes that are automodified by PAR. To see if the modified β-NAD$^+$ can homogenously modify PARP, we used human TNKS-1 in an in vitro automodification experiment with compound 2.3. In a previous work, Tan and co-workers have reported that the catalytic domain of hTNKS-1 is capable of automodification to produce PAR.$^7$ To this end, we were able to obtain the catalytic domain of hTNKS-1 for this experiment.
**Figure 2.3** Protein assays of human Tankyrase-1 (hTNKS-1) carried out with \( \beta \)-NAD\(^+\) and proparagyl-\( \beta \)-NAD\(^+\) (2.3) carried out for 90 minutes. The hTNKS-1 used did not show signs of automodification taking place. Experiment was carried out in 100 mM Tris-buffer with 10 mM MgCl\(_2\), 1 \( \mu \)M hTNKS-1, and 100 \( \mu \)M substrate. (From left to right) lane 1: Ladder, lane 2: hTNKS-1 + H\(_2\)O, lane 3: hTNKS-1 + \( \beta \)-NAD\(^+\), lane 4: hTNKS-1 + \( \beta \)-NAD\(^+\) + 2.3, lane 5: hTNKS-1 + 2.3.

The automodification experiment was carried out with hTNKS-1, unmodified \( \beta \)-NAD\(^+\), and proparagyl-\( \beta \)-NAD\(^+\) (2.3). Samples of \( \beta \)-NAD\(^+\) derivative 2.3 were obtained from Michael Lambrecht, and hTNKS-1 was incubated with \( \beta \)-NAD\(^+\) or 2.3 for 90 minutes and its results were visualized via Coomassie blue stain (Figure 2.3). Unfortunately, the hTNKS-1 catalytic domain samples used did not show signs of automodification: In figure 2, lanes 2 and 3 showed the hTNKS-1 bands moving the same distance down the gel, showing that automodification may not have taken place. Overnight incubation also failed to show visual evidence that automodification took place. Despite the lack of visual evidence that automodification took place, we cannot confirm that the catalytic domain of hTNKS-1 does not produce PAR through automodification. Since PARP processes automodification upon detection of DNA damage, it is likely that a sample of damaged DNA may need to be fed into the incubation mixture to trigger the
automodification process. It is also likely that the catalytic domain of hTNKS-1 itself is incapable of automodification and the full length hTNKS-1 may need to be used.

2.2. Summary and outlook

To investigate for means to homogenously modify PARP and produce defined length PAR, masked β-NAD\(^+\) derivatives 2.2 and 2.3 were successfully synthesized and purified. Compound 2.3 was used in an automodification experiment with hTNKS-1 to verify its ability to homogenously modify PARP. However, the results were inconclusive – the sample of hTNKS-1 failed to carry out automodification even with the standard β-NAD\(^+\) substrate that was used, suggesting that either the full-length hTNKS-1 needs to be used or a sample of damaged DNA needs to be co-injected to initiate the modification procedure.

Should this method succeed, one barrier still needs to be overcome. As shown in the previous chapter, automodification of PARP by PAR may result in branching for long-chain PAR. The masked β-NAD\(^+\) derivative used in this experiment only has the 2‘-hydroxyl group of adenosine masked. Since branching tends to occur on the 2-hydroxyl group of ribose, there may arise the need to bioorthogonally mask the 2-hydroxyl group should we attempt to synthesize longer PAR molecules (>10 ADP units). A possible course of action would be to use masking groups that are cleaved under different wavelengths of light to orthogonally protect the two hydroxyl groups in question.

Additionally, β-NAD\(^+\) derivative 2.3 could be used in the conclusive identification of number of PARP modification sites as well as their chemical nature. Methods utilized by Guy Poirier and Leung and their respective co-workers appear to be inconclusive, as there is little to no agreement in their final results of PARP modification sites.8-10 Although current reviews
generally agree that the modification occurs on glutamate, aspartate, and lysine residues, the lack of agreement among these studies necessitate the development of a method to conclusively identify the total number of and chemical nature of modification sites on PARP. If $\beta$-NAD$^+$ derivative 2.3 is used in an automodification experiment with hPARP-1 then analyzed via LC/MS, at least the number of modification sites can be identified via the mass difference between the homogenously modified PARP and unmodified PARP.

2.3. Materials and methods

2.3.1. Chemical information

*General* All reactions were run in flame or oven dried glassware under an atmosphere of dry nitrogen unless otherwise noted. Carbohydrates 8 and 13 were purchased from Carbosynth. Adenosine (6), and TBSCl were purchased from Chem Impex Intl. TMSOTf was purchased from TCI chemicals. N,N-diisopropylphosphoramidite was purchased from Alfa Aesar. All other reagents and starting materials were purchased from Sigma-Aldrich. Acetonitrile and DMF used in reactions were obtained from a solvent dispensing system. Diethyl ether was distilled from sodium metal. 4 Å molecular sieves were dried at 200°C on high vacuum overnight. Pyridine was distilled from CaH$_2$ and stored on 4 Å molecular sieves. All other reagents were of standard commercial purity and were used as received.

*Compound Analysis* Analytical thin-layer chromatography was performed on EMD Merck silica gel plates with F254 indicator. Plates were visualized with UV light (254 nm) or staining with panisaldehyde. Silica gel for column chromatography was purchased from Sorbent Technologies (40-75 μm particle size). All $^1$H, $^{13}$C, and $^{31}$P NMR spectra were recorded at 500, 125, and 202 MHz, respectively unless otherwise indicated. $^1$H and $^{13}$C NMR spectra were referenced to the residual solvent peak as reported by Fulmer et al.$^{16}$ Chemical shifts are reported in ppm and multiplicities are reported as s (singlet), d (doublet), t (triplet), q (quartet), p (pentet), h (hextet), hep (heptet), m (multiplet), and br (broad). Mass spectrometry analysis was performed by the University of Illinois Mass Spectrometry Center or by direct injection on an Agilent 6230 LC/MS TOF for samples run in negative ion mode. The LC/MS assay was
performed on the Agilent 6230 LC/MS TOF system with a 1.8 µm, 2.1x50 mm Agilent ZORBAX Eclipse Plus C18 column. Analytical HPLC analysis was performed on a Waters e2695 separations module with a Waters 2489 UV detector using a 5 µm, 4.6x150 mm Waters XBridge BEH130 HPLC column. All other C18 chromatography was performed using a Teledyne Isco CombiFlash Rf system with CombiFlash Gold columns.

**Compound 2.2:** Imidazolide 2.10 (20 mg, 38 µmol, 1.2 eq) was dissolved in dry formamide (0.3 mL) under a steady flow of dry N₂ gas, followed by addition of phosphate 2.7 (10 mg, 31 µmol, 1.0 eq), anhydrous manganese (II) chloride (6 mg, 47 µmol, 1.5 eq), and magnesium sulfate (7.5 mg, 63 µmol, 2.0 eq). The mixture was left to stir at room temperature overnight. The reaction was purified via prep-HPLC to yield pyrophosphate 2.2 as a yellowish-white solid (6 mg, 25%).

**¹H NMR (500 MHz, D₂O)** δ 9.29 (s, 1H), 9.11 (d, J = 5.9 Hz, 1H), 8.79 (d, J = 7.8 Hz, 1H), 8.20 – 8.12 (m, 1H), 8.02 (s, 1H), 7.60 (d, J = 8.1 Hz, 1H), 7.40 – 7.22 (m, 4H), 6.04 (d, J = 5.0 Hz, 1H), 5.85 (d, J = 7.3 Hz, 1H), 5.23 (d, J = 13.8 Hz, 1H), 4.64 (d, J = 13.3 Hz, 1H), 3.55 – 3.48 (m, 2H), 3.30 (t, J = 5.8 Hz, 1H), 3.10 – 3.02 (m, 1H), 2.62 – 2.58 (m, 1H).

**¹³C NMR (125 MHz, D₂O)** δ 210.84, 185.82, 168.34, 161.96, 155.41, 152.84, 151.87, 129.56, 128.79, 124.65, 105.78, 100.24, 85.16, 79.61, 77.73, 69.81, 46.85, 8.40.

**HRMS (ESI)** m/z calc’d for C₂₈H₃₃N₈O₁₆P₂ ([M+H]⁺) 799.1490, found 799.1478.
**Compound 2.3:** Imidazolide 2.12 (20 mg, 46 μmol, 1.2 eq) was dissolved in dry formamide (0.4 mL) under a steady flow of dry N₂ gas, followed by addition of phosphate 2.7 (12.5 mg, 38 μmol, 1.0 eq), anhydrous manganese (II) chloride (7.2 mg, 57 μmol, 1.5 eq), and magnesium sulfate (9.2 mg, 77 μmol, 2.0 eq). The mixture was left to stir at room temperature overnight. The reaction was purified via prep-HPLC to yield pyrophosphate 2.3 as a yellowish-white solid (10 mg, 34%).

**HRMS (ESI) m/z calc’d for C₂₄H₃₀N₇O₁₄P₂ ([M+H]⁺) 335.0644, found 702.1323.**

**Compound 2.4:** Diol 2.9 (500 mg, 1.24 mmol, 1.0 eq) was dissolved in trimethyl phosphate (3.2 mL) and cooled to 0°C in an ice bath. Phosphorous oxychloride (0.46 mL, 4.97 mmol, 4.0 eq) was added dropwise to the solution under a steady flow of dry N₂ gas. The reaction mixture was stirred for 2 hours at 0°C. The reaction was monitored every 40 minutes via LCMS. The reaction was quenched with deionized water, concentrated via rotary evaporation, and purified via C-18 chromatography to yield phosphate 2.4 as a white solid (277 mg, 46%).

**¹H NMR (500 MHz, DMSO-d₆) δ 8.31 (s, 1H), 8.12 (s, 1H), 7.99 (dd, J = 8.1, 1.3 Hz, 1H), 7.68 (d, J = 7.9 Hz, 1H), 7.62 (dd, J = 7.6, 7.5 Hz, 1H), 7.50 (dd, J = 8.0, 7.1 Hz, 1H), 7.33 (s,
2H), 6.15 (d, J = 5.4 Hz, 1H), 5.05 (d, J = 14.7 Hz, 1H), 4.91 (d, J = 14.7 Hz, 1H), 4.61 (dd, J = 5.2, 5.2 Hz, 1H), 4.44 (dd, J = (4.5, 4.5 Hz, 1H), 4.18 – 4.14 (m, 1H), 4.14 – 4.08 (m, 1H), 4.02 – 3.96 (m, 1H).

\[ ^{13} \text{C NMR (125 MHz, DMSO-d6)} \delta 208.4, 162.5, 159.7, 156.5, 153.1, 150.0, 139.9, 134.4, 129.3, 129.2, 125.1, 94.1, 86.3, 81.7, 69.6, 68.7, 28.1. \]

\[ ^{31} \text{P NMR (202 MHz, CDCl}_3 \) \delta 0.01. \]

**HRMS (ESI) m/z calc’d for C\(_{17}\)H\(_{20}\)N\(_6\)O\(_9\)P ([M+2H]\(^{+}\)) 483.1029, found 483.1032.**

Compound 2.5: Diol 2.11 (95 mg, 0.311 mmol, 1.0 eq) was dissolved in trimethyl phosphate (0.8 mL) and cooled to 0°C in an ice bath. Phosphorous oxychloride (0.11 mL, 1.24 mmol, 4.0 eq) was added dropwise to the solution under a steady flow of dry N\(_2\) gas. The reaction mixture was stirred for 2 hours at 0°C. The reaction was quenched with deionized water, concentrated via rotary evaporation, and purified via C-18 chromatography to yield phosphate 2.5 as a pale yellow solid (140 mg, quant.).

\[ ^1 \text{H NMR (500 MHz, DMSO-d6)} \delta 8.51 (s, 1H), 8.33 (s, 1H), 6.18 (d, J = 5.4 Hz, 1H), 4.55 (dd, J = 5.0, 3.8 Hz, 1H), 4.30 – 4.26 (m, 1H), 4.23 (dd, J = 6.6, 2.4 Hz, 1H), 4.12 – 4.01 (m, 2H), 3.47 (d, J = 10.7, 2H), 2.61 (t, J = 2.5 Hz, 1H). \]

**HRMS (ESI) m/z calc’d for C\(_{13}\)H\(_{15}\)N\(_5\)O\(_7\)P ([M\(^{+}\)]) 384.0709, found 384.0711.**
**Compound 2.7**: Phosphorylation of ribosyl nicotinamide (2.14) was carried out based on a procedure reported by Kaminishi et al.\(^3\) Triol 2.14 (1.4 g, 3.50 mmol, 1.0 eq) was dissolved in trimethyl phosphate (9.5 mL) in an ice bath, followed by addition of phosphorous oxychloride (1.35 mL, 14.7 mmol, 4.0 eq). The mixture was left to stir at 5\(^\circ\)C overnight. The reaction was quenched by addition of cold aqueous solution of 2N sodium hydroxide (5 mL). The reaction mixture was washed with cold 1:3 mixture of acetonitrile and diethyl ether, and the aqueous layer was subjected to an ion exchange column (Dowex 1x2 100-200 mesh, formate form, 1.5 cm x 12 cm) and eluted with water. The fractions corresponding to the product were pooled together and concentrated via rotary evaporation. The concentrated solution was purified once more via C-18 chromatography to yield phosphate 2.7 as a yellowish-white solid (200 mg, 17%).

**\(^1^H\) NMR (500 MHz, D\(_2\)O)** \(\delta 9.49 (s, 1H), 9.29 (d, J = 6.7 Hz, 1H), 8.99 (d, J = 8.2 Hz, 1H), 8.30 (dd, J = 8.2, 6.1 Hz, 1H), 6.22 (d, J = 5.5 Hz, 1H), 4.64 (dd, J = 5.0, 2.3 Hz, 1H), 4.58 (dd, J = 5.4, 2.3 Hz, 1H), 4.45 (ddd, J = 4.9, 2.4, 2.4 Hz, 1H), 4.30 (ddd, J = 12.0, 4.4, 2.3 Hz, 1H), 4.13 (ddd, J = 12.0, 4.9, 2.3 Hz, 1H).

**\(^{13}^C\) NMR (125 MHz, D\(_2\)O)** \(\delta 210.9, 146.2, 142.7, 140.0, 134.3, 128.7, 100.2, 87.8, 77.9, 71.3, 64.2, 44.0\).

HRMS (ESI) m/z calc’d for C\(_{11}\)H\(_{16}\)N\(_2\)O\(_8\)P ([M+H]\(^+\)) 335.0644, found 335.0657.
Compound 2.9: A modified version of the procedure of Chaulk et al. was followed. Adenosine (2.6) (1.0 g, 3.74 mmol, 1.0 eq) was dissolved in hot DMF (30 mL) and heated to 60°C. Once the temperature was reached, the solution was cooled to 0°C in an ice bath. A suspension of sodium hydride (195 mg, 4.86 mmol, 1.3 eq) in cold DMF (3 mL) was added to the solution and stirred for 10 minutes in an ice bath, followed by the addition of a solution of o-nitrobenzyl bromide (1.213 g, 5.61 mmol, 1.5 eq) in cold DMF (3 mL) at room temperature. The reaction mixture was stirred for an additional 5 hours at room temperature. The mixture was poured out to a 250 mL round-bottom flask containing ice-cold water (30 mL) and stirred overnight. The precipitate from the reaction mixture was filtered out and washed with ethanol (3 x 20 mL). The solid obtained was dried in vacuum and characterized through 1H NMR, 13C NMR, and HSQC/HMBC to establish that the white precipitate was diol 2.9 (903 mg, 60%).

1H NMR (500 MHz, DMSO-d6) δ 8.35 (s, 1H), 8.09 (s, 1H), 7.97 (dd, J = 8.2, 1.2 Hz, 1H), 7.62 (dd, J = 7.5, 1.8 Hz, 1H), 7.58 (ddd, J = 7.8, 7.0, 1.3 Hz, 1H), 7.49 (ddd, J = 8.4, 7.0, 1.8 Hz, 1H), 7.36 (s, 2H), 6.10 (d, J = 6.1 Hz, 1H), 5.44 (d, J = 4.8 Hz, 1H), 5.43 (dd, J = 4.5, 4.4 Hz, 1H), 5.03 (d, J = 14.7 Hz, 1H), 4.86 (d, J = 14.7 Hz, 1H), 4.59 (dd, J = 6.2, 4.7 Hz, 1H), 4.41 (ddd, J = (4.9, 4.7, 3.4 Hz, 1H), 4.04 (ddd, J = (3.5, 3.4, 3.3 Hz, 1H), 3.69 (ddd, J = 12.2, 4.3, 4.1 Hz, 1H), 3.58 (ddd, J = 12.2, 7.2, 3.6 Hz, 1H).

13C NMR (125 MHz, DMSO-d6) δ 159.7, 153.1, 152.1, 149.5, 140.3, 134.4, 129.6, 129.4, 125.0, 92.6, 87.0, 86.6, 81.8, 77.4, 69.5, 68.6, 62.0.

Compound 2.10: Phosphate 2.4 (24 mg, 49.9 μmol, 1.0 eq) was dissolved in DMSO (0.42 mL) under a steady flow of dry N2 gas at room temperature, followed by addition of aldrithiol (36 mg,
165 μmol, 3.3 eq), triphenylphosphine (43 mg, 165 μmol, 3.3 eq), and imidazole (54.3 mg, 798 μmol, 16 eq). A stock solution of triethylamine (0.5 mL) was prepared in DMSO (2.5 mL), and 0.1 mL of the stock solution was added to the reaction mixture under a steady flow of dry N\textsubscript{2} gas at room temperature. The mixture was left to stir at room temperature for 5 hours. The product was purified via C-18 chromatography to yield imidazolide 2.10 as a yellow solid (20 mg, 77%).

\textbf{1H NMR (500 MHz, CD\textsubscript{3}OD)} δ 8.31 (s, 1H), 8.14 (s, 1H), 8.05 (s, 1H), 7.89 (dd, J = 8.2, 1.3 Hz), 7.64 (dd, J = 7.8, 1.4 Hz, 1H), 7.51 (dd, J = 7.6, 1.3 Hz, 1H), 7.40 (ddd, J = 7.7, 7.4, 1.5 Hz, 1H), 7.33 (s 1H), 7.25 (s, 2H), 7.06 (s, 1H), 6.16 (d, J = 5.9 Hz, 1H), 5.13 (d, J = 14.4 Hz, 1H), 4.94 (d, J = 14.3 Hz, 1H), 4.62 (dd, J = 5.9, 4.8 Hz, 1H), 4.41 (dd, J = 5.0, 3.5 Hz, 1H), 4.21 (dd, J = 3.6, 1.8 Hz, 1H), 4.10 (ddd, J = 11.4, 5.8, 3.5 Hz, 1H), 4.04 (ddd, J = 11.4, 6.2, 3.7 Hz, 1H).

\begin{center}
\begin{tikzpicture}

\begin{scope}[scale=0.8]

% Compound 2.6
% Compound 2.11: A modified version of the procedure of Chaulk et al. was followed.\textsuperscript{4}

Adenosine (2.6) (1.0 g, 3.74 mmol, 1.0 eq) was dissolved in hot DMF (35 mL) and heated to 60°C. Once the temperature was reached, the solution was cooled to 0°C in an ice bath. A suspension of sodium hydride (195 mg, 4.86 mmol, 1.3 eq) in cold DMF (5 mL) was added to the solution and stirred for 15 minutes in an ice bath, followed by the addition of a solution of proparagyl bromide (834.2 mg, 5.61 mmol, 1.5 eq) in cold DMF (3 mL) at room temperature. The reaction mixture was stirred for an additional 5 hours at room temperature. The mixture was quenched with MeOH (3 mL) and the solvent was removed under reduced pressure. The product was purified via silica column chromatography (R\textsubscript{f} = 0.50 in 9:1 DCM:MeOH) and recrystallization from EtOH to yield compound 2.11 as pale yellow oil (381.5 mg, 31%).

\textbf{1H NMR (500 MHz, DMSO-d6)} δ 8.35 (s, 1H), 8.12 (s, 1H), 7.35 (s, 2H), 6.00 (d, J = 6.4 Hz, 1H), 5.47 (dd, J = 7.1, 4.5 Hz, 1H), 5.34 (d, J = 5.1 Hz, 1H), 4.67 (dd, J = 6.4, 4.8 Hz, 1H), 4.36
– 4.31 (m, 2H), 4.25 (dd, J = 15.9, 2.4 Hz, 1H), 4.17 (dd, J = 15.9, 2.4 Hz, 1H), 3.98 (dd, J = 6.4, 3.2 Hz, 1H), 3.65 (ddd, J = 12.1, 8.0, 4.0 Hz, 1H), 3.54 (ddd, J = 12.1, 7.1, 3.5 Hz, 1H).

**HRMS (ESI)** m/z calc’d for C₁₃H₁₆N₅O₄ ([M+H]⁺) 306.1202, found 306.1193.

**Compound 2.12:** Phosphate 2.5 (100 mg, 0.260 mmol, 1.0 eq) was dissolved in DMSO (2.6 mL) under a steady flow of dry N₂ gas at room temperature, followed by addition of aldrithiol (189 mg, 0.859 mmol, 3.3 eq), triphenylphosphine (225 mg, 0.859 mmol, 3.3 eq), imidazole (283 mg, 4.16 mmol, 16 eq) and triethylamine (91 µL). The mixture was left to stir at room temperature for 5 hours. The product was purified via C-18 chromatography to yield imidazolide 2.12 as a yellow solid (25.5 mg, 22%).

\[ ^{1}H \text{ NMR (500 MHz, CD}_3\text{OD)} \delta 8.40 (s, 1H), 8.19 (s, 1H), 8.04 (s, 1H), 8.01 (s, 1H), 7.05 (s, 1H), 6.13 (d, J = 5.8 Hz, 1H), 4.73 (t, J = 5.5 Hz, 1H), 4.41 – 4.36 (m, 1H), 4.30 (dd, J = 8.4, 2.4 Hz, 2H), 4.17 – 4.14 (m, 1H) 4.11 – 3.99 (m, 2H), 2.76 (t, J = 2.4 Hz, 1H). \]

\[ ^{31}P \text{ NMR (202 MHz, D}_2\text{O)} \delta -6.96 \]

**HRMS (ESI)** m/z calc’d for C₁₆H₁₉N₇O₆P ([M+H]⁺) 436.1134, found 436.1138.

![Chemical structure of Compound 2.8 and 2.13]
**Compound 2.13**: Tetraacetate 2.8 (2.0 g, 6.28 mmol, 1.0 eq) was dissolved in dry acetonitrile (20 mL) under a steady flow of dry N₂ gas at room temperature, followed by addition of niacinamide (920 mg, 7.54 mmol, 1.2 eq). Trimethylsilyl triflate (1.35 mL, 7.54 mmol, 1.2 eq) was added dropwise to the reaction mixture under a steady flow of dry N₂ gas at room temperature. The mixture was left to stir at room temperature for 2 hours. The product was purified via C-18 chromatography to yield triacetate 2.13 as a white foamy solid (2.91 g, 87%).

**¹H NMR (500 MHz, D₂O)** δ 9.51 (dd, J = 1.7, 1.6 Hz, 1H), 9.27 (dd, J = 5.1, 1.7 Hz, 1H), 9.05 (dd, J = 5.1, 1.4 Hz, 1H), 8.34 (dd, J = 6.9, 7.4 Hz, 1H), 6.65 (d, J = 3.8 Hz, 1H), 5.63 (dd, J = 5.4, 3.8 Hz, 1H), 5.52 (dd, J = 5.4, 5.4 Hz, 1H), 4.96 (ddd, J = 5.4, 2.7, 2.7 Hz, 1H), 4.60 (dd, J = 13.2, 2.8 Hz, 2H), 2.23 (s, 3H), 2.19 (s, 3H), 2.16 (s, 3H).

**¹³C NMR (125 MHz, D₂O)** δ 210.5, 198.6, 195.1, 172.6, 146.4, 143.3, 140.6, 128.8, 97.5, 82.8, 76.6, 69.6, 62.8, 44.0, 31.4, 20.4, 20.0.

**HRMS (ESI)** m/z calc’d for C₁₇H₂₁N₂O₈ ([M]+) 381.1298, found 381.1305.

**Compound 2.14**: Triacetate 2.13 (2.9 g, 5.47 mmol, 1.0 eq) was dissolved in methanol (50 mL) in an ice bath, followed by addition of a solution of sodium methoxide (1.477 g, 27.3 mmol, 5.0 eq) dissolved in ice-cold methanol (50 mL). The mixture was left to stir at -20°C overnight. The reaction was quenched by addition of glacial acetic acid (2 mL) and the product was purified via C-18 chromatography to yield triol 2.14 as a white foamy solid (2.70 g, quant.).

**¹H NMR (500 MHz, D₂O)** δ 9.62 (dd, J = 1.5, 1.3 Hz, 1H), 9.28 (ddd, J = 6.5, 1.3, 1.3 Hz, 1H), 8.99 (dd, J = 8.0, 1.5, 1.3 Hz, 1H), 8.29 (dd, J = 8.1, 6.3 Hz, 1H), 6.26 (d, J = 4.4 Hz, 1H), 4.52 (t, J = 4.7 Hz, 1H), 4.50 (ddd, J = 3.5, 3.5, 2.9 Hz, 1H), 4.37 (dd, J = 4.6, 4.6 Hz, 1H), 4.06 (dd, J = 12.9, 2.9 Hz, 1H), 3.91 (dd, J = 12.9, 3.6 Hz, 1H).
$^{13}$C NMR (125 MHz, D$_2$O) $\delta$ 210.6, 145.8, 142.9, 140.6, 128.6, 100.1, 87.9, 77.6, 70.0, 60.4, 44.0.

**HRMS (ESI)** m/z calc’d for C$_{11}$H$_{15}$N$_2$O$_5$ ([M]$^+$) 255.0981, found 255.0990.

2.3.2. Biological experiment

*Human Tankyrase-1 (hTNKS-1) automodification experiment*  Concentrated TNKS-1 stock solutions were diluted to 1 µM. Next, to four 0.5 mL tubes were added 6 µL of PARP activity buffer (100 mM Tris, 10 mM MgCl$_2$), 1 µL of 1 mM TCEP, and 2 µL of the diluted hTNKS-1. The following treatments were implemented to each tube: **Tube 1**: 1 µL of deionized water; **tube 2**: 1 µL of a 100 µM solution of β-NAD$^+$; **tube 3**: 1 µL of a 1:1 mixture of the prepared 100 µM β-NAD$^+$ solution and the 100 µM solution of **2.3**; **tube 4**: 1 µL of a 100 µM solution of **2.3**. The tubes were incubated at room temperature for 90 minutes then analyzed via SDS-PAGE on a 4~20% gel. The gel was stained by Coomassie Blue and the results visualized by BIO-RAD Molecular Imager Gel-Doc™ XR+. This process was repeated a second time with a longer incubation time (17 hr).
2.3.3. Spectra
2.4. References

CHAPTER 3

INVESTIGATION OF CELL-PERMEABLE PARG INHIBITORS

Along with PARP inhibition, PARG inhibition has been suggested as a plausible means to induce cell death via PARthanatos;\(^1,2\) however, investigation of PARG inhibitors is stymied by the lack of cell-permeable and specific PARG inhibitors. ADP-HPD, the most potent and specific PARG inhibitor known in literature,\(^3\) lacks the lipophilicity to penetrate cell membranes. Jacobson and co-workers previously used an alkylated derivative of ADP-HPD in the investigation of the SAR of ADP-HPD; however, cell-permeability assays were not carried out on the molecule.\(^4\)

Here we report the strategy of employing hydrophobic masking groups to the pyrophosphate moiety of ADP-HPM to examine its cell-permeability while maintaining potent inhibition of PARG. The pyrophosphate is coupled to a lipophilic alanine ester to enhance cell penetration. Once the molecule enters the cytosol, the alanine ester is enzymatically removed to expose the deprotected ADP-HPD.

3.1. Masking group strategies

Masking group strategies have been extensively employed in the literature for investigations of phosphate, diphosphate, triphosphate, and pyrophosphate drugs.\(^5-8\) Among the masking groups, amino-acid-based masking groups have garnered considerable interest due to their relative stability to other masking groups and nontoxic byproduct of amino acids.\(^9\) It is utilized for commercially approved prodrugs such as Sovaldi.\(^10\) The lipophilicity of the prodrug can be tuned by changing the ester of the amino acid.
Scheme 3.1. Enzymatic cleavage of amino-acid-based masking groups on phosphate prodrugs. The enzymes esterase and HINT-1 are involved in the enzymatic removal of the amino-acid-ester masking groups. Shown here are the mechanisms of aryl phosphoramidate prodrug activation (A) and diamide prodrug activation (B).

Scheme 3.1 shows the mechanism of activation of phosphoramidate prodrugs. The amino acid ester’s lipid is cleaved by esterase, leading to a spontaneous loss of the neighboring masking group – phenol in the case of aryl phosphoramidates (Scheme 3.1A) and one of the two amino acids in the case of diamides (Scheme 3.1B). The remaining amino acid subsequently cyclizes into the phosphate to form a heteroatomic pentacyclic intermediate, which is hydrolyzed. Finally, the enzyme HINT1 enzymatically removes the remaining amino acid to reveal the deprotected phosphate.

While most of the reported masking groups are utilized in phosphate or diphosphate prodrugs, pyrophosphate masking groups have emerged in the recent years. In particular, Gray and co-workers have been able to utilize the amino-acid-based masking group strategy in their investigation of a K-Ras inhibitor (3.1). Based on the structure of the K-ras inhibitor and the original structure of ADP-HPM (3.2) shown in figure 3.1, here we propose an amino-acid-ester-masked ADP-HPM (3.3) that can be used to examine the cell-permeability of the prodrug. A possible mechanism of activation of the pyrophosphate prodrug is shown in scheme 3.2. Similar to scheme 3.1, esterase should cleave the lipophilic ester moiety, followed by removal of the
amino acid by phosphoramidase HINT-1. To ensure the stability of the P—N bond of the phosphoramidate functional group, a stability assay will be carried out via LC/MS to monitor its cleavage over a period of time.

Figure 3.1. Structures of K-ras inhibitor SML-10-70-1, ADP-HPM, and masked-ADP-HPM. Based on the structure of K-ras inhibitor SML-10-70-1 (3.1) developed by Gray and co-workers, a similar masking group will be utilized on the pyrophosphate moiety of ADP-HPM (3.2) to produce masked-ADP-HPD (3.3)

Scheme 3.2. Possible mechanism of enzymatic cleavage of amino acid ester masking groups on pyrophosphate prodrugs. Similar to the removal of masking groups on aryl phosphoramidate or diamide prodrugs, the enzymes esterase and HINT-1 carry out a stepwise removal of the amino acid ester masking group to expose the deprotected pyrophosphate moiety.

3.2. Synthesis and evaluation of masked ADP-HPM

3.2.1. Synthesis of protected phosphoramidate 3.5

The retrosynthetic analysis is shown below in scheme 3.3. The pyrophosphate will be synthesized from commercially available AMP morpholide 3.4 and protected phosphoramidate
3.5. The protected phosphoramidate will be synthesized from H-phosphonate 3.7 and L-alanine ester 3.6 following a modified procedure used by Gray and Zhao and their respective co-workers. H-phosphonate 3.7 can be synthesized from alcohol 3.10 following procedures by Zhao and co-workers, and alcohol 3.10 can be produced from a series of protection and reduction reactions of commercially available proline derivative 3.11.

Scheme 3.3. Retrosynthetic analysis of masked-ADP-HPM. The molecule can be synthesized from a pyrophosphate coupling of a commercially available source of activated AMP morpholide 3.4 and protected phosphoramidate 3.5, which in turn can be synthesized from building blocks of alanine ester 3.6 and trans-L-4-hydroxyproline methyl ester (3.11).

Scheme 3.4 shows the overall progress of the synthesis of 3.5. The benzyl-group-based protecting groups were chosen since removal of acid- or base-labile protecting groups in subsequent steps had possibilities of cleaving the P—N bond of the phosphoramidate. The protection of the secondary amine of 1-trans-4-hydroxyproline methyl ester (3.11) was carried out with benzyl chloroformate and N,N-diisopropylethylamine in DCM. Benzylation of the hydroxyl group was carried out with benzyl bromide and sodium hydride in THF. Tetrabutylammonium iodide (TBAI) was added to enhance the rate of conversion. Finally,
reduction of the ester was carried out by lithium borohydride, a milder reducing agent than lithium aluminum hydride, which could react with the Cbz-protecting group, but stronger agent than sodium borohydride to provide a quantitative yield of alcohol 3.10. The formation of H-phosphonate 3.7 was carried out following a modified procedure reported by Zhao and co-workers.\(^\text{10}\)

**Scheme 3.4. Synthesis of protected phosphoramidate 3.5.**
Following the synthesis of protected alanine ester 3.14 and its subsequent deprotection, synthesis of phosphoramidate 3.5 was carried out following the procedure utilized by Gray and co-workers. The synthesis began with a modified Atherton-Todd reaction also utilized by Zhao and co-workers, followed by deprotection of the fluorenlymethy group with triethylamine in acetonitrile. While the two original papers use piperidine to remove the protecting group, triethylamine was utilized for this reaction under suspicion that the piperidinium salt may interfere with the subsequent pyrophosphate coupling step, discussed in section 3.2.3.

![Figure 3.2](image_url)

**Figure 3.2. Monitoring of P—N bond stability on phosphoramidate 3,5.** 1 mg samples of phosphoramidate 3.5 were dissolved in 20% CH₃CN in deionized H₂O (v/v) and monitored by LC/MS over a period of 24 hours. The phosphoramidate was detected at an m/z of 603 for up to 24 hours in solution, proving that the compound was stable to hydrolysis.
3.2.2. Masking group stability assay

With phosphoramidate 3.5 in hand, we monitored its stability by LCMS. The phosphoramidate was dissolved in a solution of 20% acetonitrile in deionized water and mass spectra were taken at different time intervals. As shown in figure 3.2, the compound was detected in solution for up to 24 hours and longer, indicating that the P—N bond was stable in aqueous solution without undergoing hydrolysis. Furthermore, the overnight deprotection of the fluorenlylmethyl group with triethylamine in acetonitrile following the Atherton-Todd coupling between the alanine ester and the H-phosphonate shows that the P—N bond can withstand mildly to moderately basic conditions for up to 24 hours.

3.2.3. Completing the synthesis of masked ADP-HPM

Much like the pyrophosphate coupling step as discussed in chapter 2, the pyrophosphate coupling step of masked ADP-HPM was the toughest step in the synthesis. Attempts to carry out the coupling are outlined in scheme 3.5. Initially the pyrophosphate reaction was carried out under conditions similar to that of Gray and co-workers, with 5-ethylthio-1H-tetrazole as the catalyst and piperidinium as the counter-ion. However, no reaction occurred under these conditions. When the pyrophosphate coupling was attempted with similar reaction conditions using MnCl₂ in anhydrous formamide as used in chapter 2, a very low conversion rate was observed. In expectations that changing the lewis acid could result in a better conversion rate, conditions utilized by Vincent and co-workers, with MgCl₂ in dry DMF, and conditions by Lambrecht and co-workers, with ZnCl₂ in DMF, were employed. While both conditions resulted in slightly higher conversion rates than when MnCl₂ was used, the conversion rate was still below 10%, which was not considered synthetically useful.
Under the assumption that the piperidinium counter-ion may have interfered with the pyrophosphate coupling, we synthesized phosphoramidate 3.5 without using piperidine to deprotect the fluorenlylmethyl group on the phosphoramidate after the Atherton-Todd coupling. Then, using a commercially available AMP-morpholide (3.4) from Sigma-Aldrich, the pyrophosphate coupling was carried out as done by Gray and co-workers. This time the pyrophosphate coupling was a success, with a conversion rate of over 50%. The pyrophosphate was purifiable with C-18 chromatography.

**Scheme 3.5. Pyrophosphate coupling of 3.5.** AG = Activating Group. Conditions of the coupling are outlined in table 3.1

<table>
<thead>
<tr>
<th>Counter-ion of 3.5</th>
<th>Activating group</th>
<th>Conditions</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperidinium ion</td>
<td>Morpholine</td>
<td>5-ethylthio-1H-tetrazole in pyridine r.t., 3 hr&lt;sup&gt;6&lt;/sup&gt;</td>
<td>No reaction</td>
</tr>
<tr>
<td>Et&lt;sub&gt;3&lt;/sub&gt;NH&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Imidazole</td>
<td>MnCl₂, MgSO₄, anhydrous formamide r.t., 18 hr</td>
<td>&lt;5% conversion</td>
</tr>
<tr>
<td>Et&lt;sub&gt;3&lt;/sub&gt;NH&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Imidazole</td>
<td>MgCl₂, anhydrous DMF r.t., 24 hr&lt;sup&gt;10&lt;/sup&gt;</td>
<td>&lt;10% conversion</td>
</tr>
<tr>
<td>Et&lt;sub&gt;3&lt;/sub&gt;NH&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Imidazole</td>
<td>ZnCl₂, anhydrous DMF r.t., 72 hr&lt;sup&gt;11&lt;/sup&gt;</td>
<td>&lt;10% conversion</td>
</tr>
<tr>
<td>Et&lt;sub&gt;3&lt;/sub&gt;NH&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Morpholine</td>
<td>5-ethylthio-1H-tetrazole in pyridine r.t., 16 hr&lt;sup&gt;6&lt;/sup&gt;</td>
<td>&gt;50% conversion, 54% yield</td>
</tr>
</tbody>
</table>

**Table 3.1. Pyrophosphate coupling conditions**
The synthesis of 3.3 was finalized with hydrogenation to safely remove the benzyl and benzyloxy carbonyl groups. Conditions for the hydrogenation were adopted from a procedure used by Wanner and co-workers. After 3 days of stirring under constant positive pressure of gaseous hydrogen, protected pyrophosphate 3.15 was converted into pyrophosphate 3.3. Initially there was a minor setback where we obtained 2:2:1 ratio of starting material 3.15, partially deprotected product, and fully deprotected product (desired product) 3.3. The results weren’t too surprising since there are reports for similar substrates that the hydrogenolysis of the benzyl group proceeds more slowly than that of the benzyloxy carbonyl group. Different hydrogenolysis conditions need to be sought to ensure efficient removal of both protecting groups.

Scheme 3.6. Hydrogenolysis of pyrophosphate 3.15. Conditions were adopted from the works of Wanner and co-workers.

3.2.4. Masking group cleavage assay with HINT-1

Even if masked ADP-HPM 3.3 were able to penetrate the cell membrane, if its masking group of alanine ester cannot be cleaved in the cell, it cannot achieve the desired effects as a PARG inhibitor. Histidine Triad Nucleotide-binding Protein 1 (HINT-1) is a phosphoramidase that has been reported to cleave amino acids from phosphates. To see if the masking group of modified ADP-HPM 3.3 can be cleaved by HINT-1, we used human HINT-1 in an in vitro cleavage assay with compound 3.3.
Figure 3.3. Monitoring of masking group cleavage by HINT-1. The assay was based off of a similar study carried out by Bieganowski and co-workers.\textsuperscript{16} While some amount of ADP-HPM was detected, the major species in the reaction solution were starting material (blue, m/z = 708) and AMP (red, m/z = 345). Not shown in the spectrum are traces of hydroxyproline phosphoramidate 3.16 (m/z = 379) due to lack of fluorescence.

Scheme 3.7. Cleavage of the pyrophosphate by HINT-1. It is likely that the bulky alanine ester prevented HINT-1’s cleavage of the amino acid and instead AMP was cleaved from the pyrophosphate.
The experiment was based off a similar assay carried out by Bieganowski and co-workers.\textsuperscript{16} The cleavage assay was carried out with hHint-1, masked ADP-HPM 3.3, both in a PIPES-based buffer. Varying amounts of hHINT-1 were incubated with 3.3 for 5 hours and their results were analyzed via LC/MS (Figure 3.3). While we were able to detect some amount of deprotected ADP-HPM in solution, it appeared that most of 3.3 were cleaved into AMP and hydroxyproline phosphoramidate 3.16 (Scheme 3.7), both of which were detected by LC/MS. The fluorescence peaks of 3.16 are not shown in figure 3.3 due to the molecule’s innate lack of chromophore functional groups. Given that HINT-1 has only been reported to cleave ammonia, short-chain amines, and amino acid from AMP, it is likely that the bulky group of the alanine ester of pyrophosphate 3.3 prevented HINT-1 from cleaving it successfully, and instead HINT-1 cleaved AMP from the substrate. It is also likely that the long-chain ester group needs to be removed by an esterase to convert it into a suitable substrate for HINT-1.

3.3. Summary and future directions

As an alternative to PARP inhibition for genotoxic chemotherapy, PARG inhibition has generated considerable interest in the field of chemical biology; however, the lack of cell-permeable and potent PARG inhibitors stymies any further progress in this field. To overcome this barrier, we have proposed the design of masked derivatives of ADP-HPM with hydrophobic groups to enhance its cell permeability. To this end, we successfully synthesized the desired pyrophosphate 3.3 over 10 steps, highlighting the synthetic accessibility of the test molecule. In order to test its efficacy, enzymatic assays need to be carried out: First, we must investigate that the hydrophobic masking group can be removed reliably from the pyrophosphate moiety by carboxyesterase, an enzyme that can cleave esters from amino acids, and phosphoramidase.
HINT-1. Once it is shown that the enzymes can remove the masking group, whole cell CETSA analysis can be carried out to measure the potency of the masked ADP-HPD.

3.4. Materials and methods

3.4.1. General chemical information

*General*  All reactions were run in flame or oven dried glassware under an atmosphere of dry nitrogen unless otherwise noted. 9-fluorenylmethanol (FmOH) was purchased from Chem Impex Intl. EDAC and 5-ethylthio-1-H-tetrazole were purchased from TCI chemicals. Hydrochloric acid was purchased from Fisher Scientific. Compressed hydrogen gas was purchased from Matheson Tri-Gas. All other reagents and starting materials were purchased from Sigma-Aldrich. Acetonitrile and THF used in reactions were obtained from a solvent dispensing system. Pyridine was stored on 4 Å molecular sieves that were dried quickly under high vacuum. All other reagents were of standard commercial purity and were used as received.

*Compound Analysis*  Analytical thin-layer chromatography was performed on EMD Merck silica gel plates with F254 indicator. Plates were visualized with UV light (254 nm) or staining with p-anisaldehyde. Silica gel for column chromatography was purchased from Sorbent Technologies (40-75 μm particle size). All $^1$H, $^{13}$C, and $^{31}$P NMR spectra were recorded at 500, 125, and 202 MHz, respectively unless otherwise indicated. $^1$H and $^{13}$C NMR spectra were referenced to the residual solvent peak as reported by Fulmer et al. Chemical shifts are reported in ppm and multiplicities are reported as s (singlet), d (doublet), t (triplet), q (quartet), p (pentet), h (hextet), hep (heptet), m (multiplet), and br (broad). Mass spectrometry analysis was performed by the University of Illinois Mass Spectrometry Center. The LC/MS assay was performed on the Agilent 6230 LC/MS TOF system with a 1.8 μm, 2.1x50 mm Agilent ZORBAX Eclipse Plus C18 column. Analytical HPLC analysis was performed on a Waters e2695 separations module with a Waters 2489 UV detector using a 5 μm, 4.6x150 mm Waters XBridge BEH130 HPLC column. All other C18 chromatography was performed using a Teledyne Isco CombiFlash Rf system with CombiFlash Gold columns.
**Abbreviations used**

Abbreviations used

**Compound 3.3:** Protected pyrophosphate 3.15 (25 mg, 26.8 μmol, 1 eq) was dissolved in methanol (0.32 mL), followed by the addition of concentrated HCl (2.5 μL, 30.3 μmol, 1.13 eq). 10% palladium on carbon (5 mg) was added to the solution and left to stir under positive pressure of hydrogen gas at room temperature for 3 days. The solid was filtered and the solvent was removed via rotary evaporation and the product was purified via preparatory HPLC to yield pyrophosphate 3.3 as clear oil (1 mg, 5%).

$^31$P NMR (202 MHz, D$_2$O) $\delta$ -0.82, -1.04, -10.54.

HRMS (ESI) m/z calc’d for C$_{26}$H$_{46}$N$_7$O$_{12}$P$_2$ ([M$^-$]) 710.2680, found 710.2664.

**Compound 3.5:** Compound 115 was synthesized via a modified procedure used by Gray and co-workers$^6$. Specifically, triethylamine (0.93 mL, 6.67 mmol, 6.0 eq) and carbon tetrachloride (1.7 mL) was added into a solution of alanine ester 3.6 (268 mg, 1.33 mmol, 1.2 eq) in anhydrous
THF (16.8 mL) at 0°C. A solution of H-phosphonate 3.7 (645 mg, 1.11 mmol, 1.0 eq) dissolved in a solution of anhydrous THF (6.4 mL) was added to the reaction mixture at 0°C and gradually warmed up to room temperature. The reaction mixture was left to stir at room temperature for 3 hours. The solvent was removed via rotary evaporation and the resulting slurry was redissolved in DCM (50 mL), washed with 0.1 M HCl (3 x 30 mL), and dried over Na₂SO₄. The solvent was removed via rotary evaporation and the resultant slurry was redissolved in a 1:2 mixture of triethylamine:acetonitrile (33 mL). The mixture was left to stir at room temperature for 17 hours. After removal of the solvent via rotary evaporation, the product was purified via silica column chromatography (Rf = 0.18 in 9:1 DCM:Methanol) to yield compound 3.5 as yellow oil (182 mg, 27%).

**¹H NMR (500 MHz, CD₃CN)** δ 7.40 – 7.25 (m, 10H), 5.15 – 5.00 (m, 2H), 4.57 – 4.38 (m, 2H), 4.20 – 4.10 (m, 1H), 4.07 – 3.85 (m, 3H), 3.84 – 3.55 (m, 3H), 3.43 – 3.33 (m, 1H), 2.16 – 2.07 (m, 2H), 1.70 – 1.60 (m, 1H), 1.34 – 1.22 (m, 8H), 0.91 – 0.84 (m, 6H).

**¹³C NMR (125 MHz, CD₃CN)** δ 176.50, 155.92, 139.65, 138.21, 129.42, 129.27, 128.79, 128.62, 128.56, 128.43, 78.00, 71.55, 69.18, 68.11, 67.37, 53.26, 51.49, 46.35, 37.69, 34.78, 34.15, 20.51, 17.70, 14.72.

**³¹P NMR (202 MHz, CD₃CN)** δ 5.00.

**HRMS (ESI) m/z calc’d C₃₁H₄₆N₂O₈P ([M+H]⁺) 605.2992, found 605.2991.**

**Compound 3.6:** Boc-protected L-alanine ester 3.14 (500. mg, 1.66 mmol, 1.0 eq) was dissolved in ethyl acetate (3 mL), followed by addition of 4 M dioxane solution of HCl (2.9 mL, 11.6 mmol, 7.0 eq). The reaction mixture was left to stir at room temperature for 2 hours, and then the solvents were removed via rotary evaporation. The product was used in subsequent steps without further purification.
Compound 3.7: Compound 109 was synthesized via a modified procedure used by Zhao and co-workers. Specifically, A solution of 9-fluorenemethanol (759 mg, 3.87 mmol, 1.1 eq) dissolved in anhydrous pyridine (10 mL) was added into a solution of diphenyl phosphite (0.88 mL, 4.57 mmol, 1.3 eq) in anhydrous pyridine (10 mL) and stirred at 0°C for 30 minutes. A solution of alcohol 3.10 (1.20 g, 3.52 mmol, 1.0 eq) dissolved in anhydrous pyridine (7 mL) was added to the reaction mixture at 0°C and heated up to 45°C. The reaction mixture was left to stir at 45°C for 1 hour. The solvent was removed via rotary evaporation and the product was purified via silica column chromatography (Rf = 0.31 in 1:1 ethyl acetate:hexanes) to yield compound 3.7 as yellow oil (1.34 g, 67%).

$^{1}$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.51 (dd, J = 1.7, 1.6 Hz, 1H, H-6), 9.27 (dd, J = 5.1, 1.7 Hz, 1H, H-9), 9.05 (dd, J = 5.1, 1.4 Hz, 1H, H-7), 8.34 (dd, J = 6.9, 7.4 Hz, 1H, H-8), 6.65 (d, J = 3.8 Hz, 1H, H-1), 5.63 (dd, J = 5.4, 3.8 Hz, 1H, H-2), 5.52 (dd, J = 5.4, 5.4 Hz, 1H, H-3), 4.96 (ddd, J = 5.4, 2.7, 2.7 Hz, 1H, H-4), 4.60 (dd, J = 13.2, 2.8 Hz, 2H, H-5), 2.23 (s, 3H, Ac-12), 2.19 (s, 3H, Ac-11), 2.16 (s, 3H, Ac-10).

$^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 141.66, 128.76, 128.31, 128.27, 128.24, 128.08, 127.89, 127.83, 127.56, 127.54, 127.51, 127.48, 127.45, 125.29, 120.37, 71.28, 67.61, 67.32, 67.21, 56.49, 52.49, 48.40, 48.35, 34.02.

$^{31}$P NMR (202 MHz, CDCl$_3$) $\delta$ 5.43.

HRMS (ESI) m/z calc’d for C$_{34}$H$_{35}$NO$_6$P ([M+H]$^+$) 584.2202, found 584.2202.
**Compound 3.10:** Ester 3.13 (8.28 g, 21.3 mmol, 1.0 eq) was dissolved in dry THF (185 mL) in an ice bath, followed by addition of Lithium borohydride (1.5 g, 71.0 mmol, 3.3 eq) at 0°C. The solution was left to stir for 30 minutes. The reaction was allowed to warm up to room temperature and stirred for additional 2 hours. The reaction was quenched by addition of 1 M HCl until the quenching did not produce additional gas, and the reaction mixture was diluted with deionized water (30 mL). The aqueous solution was extracted with ethyl acetate (3 x 200 mL), and the combined organic fractions were washed with water (200 mL) and brine (200 mL). The organic fraction was dried over Na$_2$SO$_4$ and concentrated via rotary evaporation. The product was purified via silica column chromatography ($R_f=0.37$ in 1:1 ethyl acetate:hexanes) to yield compound 3.10 as clear oil (6.57 mg, quant.).

$^1$H NMR (500 MHz, CDCl$_3$) δ 7.38 – 7.27 (m, 10H), 5.16 (d, J = 12.7 Hz, 1H), 5.12 (d, J = 13.1 Hz, 1H), 4.53 – 4.45 (m, 2H), 4.22 – 4.16 (m, 1H), 4.10 – 4.05 (m, 1H), 3.81 – 3.74 (m, 2H), 3.71 – 3.64 (m, 1H), 3.61 (dd, J = 12.0, 7.0 Hz, 1H), 3.47 (dd, J = 12.2, 4.4 Hz, 1H), 2.26 – 2.19 (m, 1H), 1.72 – 1.64 (m, 1H).

$^{13}$C NMR (125 MHz, CDCl$_3$) δ 128.82, 128.76, 128.42, 128.36, 128.27, 128.15, 128.05, 127.93, 127.84, 127.60, 76.50, 71.38, 71.10, 67.59, 66.72, 66.18, 59.98, 59.78, 52.98, 34.62.

HRMS (ESI) m/z calc’d for C$_{20}$H$_{23}$NO$_4$Na ([M+Na]$^+$) 364.1525, found 364.1518.

**Compound 3.12:** L-4-hydroxyproline methyl ester hydrochloride (3.11, 1.5 g, 8.26 mmol, 1.0
eq) and diisopropylethylamine (3.2 mL, 18.37 mmol, 2.2 eq) were dissolved in DCM (40 mL) at 0°C and stirred for 10 minutes. Benzyl chloroformate (1.4 mL, 9.91 mmol, 1.2 eq) was added to the reaction mixture which was left to stir for 6 hours at 0°C. The reaction mixture was quenched with saturated aqueous solution of ammonium chloride (30 mL) and extracted with DCM (2 x 50 mL). The combined organic fractions were washed with deionized water (30 mL) and brine (30 mL), dried over Na₂SO₄, and concentrated via rotary evaporation. The product was purified via silica column chromatography (Rf = 0.42 in 3:1 ethyl acetate:hexanes) to yield compound 3.12 as clear oil (2.169 g, 94%).

\[ ^1H \text{ NMR (500 MHz, CDCl}_3 \] \( \delta 7.38 - 7.29 \) (m, 5H), 5.20 – 5.04 (m, 2H), 4.56 – 4.48 (m, 2H), 3.77 and 3.56 (s, 3H), 3.73 – 3.61 (m, 2H), 2.37 – 2.26 (m, 1H), 2.14 – 2.07 (m, 1H).

\[ ^{13}C \text{ NMR (125 MHz, CDCl}_3 \] \( \delta 173.58, 155.00, 136.43, 128.68, 128.31, 128.06, 70.22, 69.44, 67.62, 58.01, 55.45, 54.88, 52.44, 39.33, 38.61.\]

HRMS (ESI) m/z calc’d for C₁₄H₁₇NO₅Na ([M+Na]⁺) 302.1004, found 302.1007.

**Compound 3.13:** Secondary alcohol 3.12 (350 mg, 1.25 mmol, 1.0 eq) was dissolved in dry THF (5 mL), followed by addition of sodium hydride (60 mg, 60% dispersion in mineral oil, 1.50 mmol, 1.2 eq) at 0°C. The solution was left to stir for 15 minutes. Benzyl bromide (0.18 mL, 1.50 mmol, 1.2 eq) and tetrabutylammonium iodide (19 mg, 50.1 μmol, 0.04 eq) were added to the reaction mixture, which was left to stir for an additional 1 hour at 0°C. The reaction was allowed to warm up to room temperature and stirred for an additional 1 hour, upon which it was diluted with ethyl acetate (5 mL) and washed with water (5 mL) and brine (5 mL). The organic fraction was dried over Na₂SO₄ and concentrated via rotary evaporation. The product was purified via silica column chromatography (Rf = 0.45 in 4:6 ethyl acetate:hexanes) to yield compound 3.13 as pale-yellow oil (151.6 mg, 33%).
$^1$H NMR (500 MHz, CDCl$_3$) δ 7.38 – 7.18 (m, 10H), 5.22 – 5.00 (m, 3H), 4.56 – 4.42 (m, 3H), 4.24 – 4.17 (m, 1H), 3.76, 3.67, and 3.54 (s, 3H), 3.71 – 3.64 (m, 1H), 2.47 – 2.38 (m, 1H), 2.13 – 2.06 (m, 1H).

HRMS (ESI) m/z calc’d for C$_{21}$H$_{24}$NO$_5$ ([M+H]$^+$) 370.1649, found 370.1658.

**Compound 3.14:** Boc-protected L-alanine (3.8, 1.60g, 8.48 mmol, 1.0 eq) was added into a solution of alcohol 3.9 (1.33 mL, 8.48 mmol, 1.0 eq) dissolved in DCM (16.8 mL), followed by addition of EDAC hydrochloride (1.95 g, 10.2 mmol, 1.2 eq) and DMAP (104 mg, 0.851 mmol, .10 eq). The reaction mixture was left to stir at room temperature for 23 hours, then diluted with ethyl acetate (100 mL) and washed with saturated aqueous solution of sodium bicarbonate (3 x 100 mL) and brine (2 x 100 mL). The organic layer was collected, dried over Na$_2$SO$_4$, and concentrated via rotary evaporation. The product was purified via silica column chromatography ($R_f$ = 0.95 in 100% ethyl acetate) to yield compound 3.14 as clear oil (2.15 g, 84%).

$^1$H NMR (500 MHz, CDCl$_3$) δ 5.05 (bs, 1H), 4.35 – 4.27 (m, 1H), 4.07 (dd, J = 10.9, 5.8 Hz, 1H), 4.01 (dd, J = 10.9, 5.6 Hz, 1H), 1.67 (ttt, J = 6.0, 5.8, 5.8 Hz, 1H), 1.44 (s, 9H), 1.38 (d, J = 7.2 Hz, 3H), 1.35 – 1.23 (m, 8H), 0.89 (t, J = 6.6 Hz, 6H).

$^{13}$C NMR (125 MHz, CDCl$_3$) δ 212.31, 107.83, 68.26, 49.50, 37.08, 33.73, 33.68, 28.59, 20.08, 19.12, 14.60.

HRMS (ESI) m/z calc’d for C$_{16}$H$_{31}$NO$_4$Na ([M+Na]$^+$) 324.2151, found 324.2146.
**Compound 3.15**: Adenosine 5’-monophosphate morpholinate 3.4 (20 mg, 28.2 μmol, 1.3 eq, from Sigma-Aldrich A1127-1G) was dissolved in anhydrous pyridine (0.22 mL), followed by the addition of a solution of phosphoramidate 3.5 (26 mg, 36.6 μmol, 1.3 eq) in anhydrous pyridine (0.22 mL). 5-ethylthio-1H-tetrazole (11 mg, 84.5 μmol, 3.0 eq) was added to the solution and left to stir with the reaction mixture at room temperature for 16 hours. The solvent was removed via rotary evaporation and the product was purified via C-18 chromatography to yield pyrophosphate 3.15 as a white foamy solid (15 mg, 58%).

\[ \text{1H NMR (500 MHz, CD}_3\text{OD)} \delta 8.52 \text{ (s, 1H), 8.19 (s, 1H), 7.40 – 7.20 (m, 10H), 6.09 (d, J = 7.5 Hz, 1H), 5.13 – 5.09 (m, 1H), 4.53 – 4.38 (m, 3H), 4.20 (dd, J = 10.9, 5.7 Hz, 2H), 4.10 (dd, J = 10.9, 5.6 Hz, 2H), 3.73 (dd, J = 5.7, 3.8 Hz, 2H), 3.40 (dd, J = 5.7, 3.7 Hz, 2H), 2.32 – 2.16 (m, 2H), 1.96 – 1.90 (m, 2H), 1.85 – 1.79 (m, 2H), 1.53 (d, J = 7.1 Hz, 3H), 1.40 – 1.25 (m, 8H), 0.93 – 0.86 (m, 6H).} \]

\[ \text{13C NMR (125 MHz, CD}_3\text{OD)} \delta 212.60, 163.91, 143.22, 141.65, 128.75, 128.39, 128.33, 128.30, 128.25, 128.22, 128.06, 127.92, 127.86, 127.82, 127.54, 127.51, 127.49, 127.46, 127.43, 125.27, 120.38, 120.35, 120.31, 76.65, 71.25, 67.54, 67.31, 67.19, 56.42, 52.43, 48.38, 48.33, 35.19, 33.97.} \]

\[ \text{31P NMR (202 MHz, CD}_3\text{OD)} \delta -1.10, -1.79, -10.67.} \]

**HRMS (ESI)** m/z calc’d for C_{41}H_{56}N_{12}O_{14}P_{2} ([M]') 932.3360, found 932.3340.
3.4.2. Biological experiment

*HINT-1 masking group cleavage experiment* The HINT-1 cleavage assay was carried out according to protocols adopted by Bieganowski and co-workers.\textsuperscript{14} Five 1 mL tubes were prepared with 50 µL reaction mixtures containing HINT-1 activity buffer (20 mM Na-PIPES, 0.5 mM MgCl\textsubscript{2}) and substrate 3.3 (50 µM). The following treatments were implemented to each tube: **Tube 1**: 1 µg of HINT-1; **tube 2**: 0.33 µg of HINT-1; **tube 3**: 0.1 µg of HINT-1; **tube 4**: 0.033 µg of HINT-1; **tube 5**: No enzyme. The tubes were incubated at 37°C for 5 hours then analyzed via LC/MS.
3.4.3. Spectra

![Spectra Diagram](image)

**Chemical Structures:**

1. **3.12**

   - Cbz
   - HO
   - OMe

   ![Chemical Structure Image](image)
3.7
3.5. References