Synthesis, purification, and characterization of the potent inhibitor of inosine monophosphate dehydrogenase: oxanosine monophosphate

Master’s Thesis

Presented to
The Faculty of the Graduate School of Arts and Sciences
Brandeis University
Department of Biochemistry
Lizbeth Hedstrom, Advisor

In Partial Fulfillment of the Requirements for
Master’s Degree

by
Philip Braunstein

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ABSTRACT

Synthesis, purification, and characterization of the potent inhibitor of inosine monophosphate dehydrogenase: oxanosine monophosphate

A thesis presented to the Biochemistry Department

Graduate School of Arts and Sciences
Brandeis University
Waltham, Massachusetts

By Philip Braunstein

Oxanosine monophosphate (OxMP) is a novel nucleotide and one of the lesions generated from reactive nitrogen species (RNS) damage of free nucleotides. Inosine monophosphate dehydrogenase (IMPDH) catalyzes the rate-limiting step of guanine nucleotide biosynthesis. IMPDH is a target for immunosuppressive and antiviral medications. IMPDH is also a promising drug target for cancers and parasitic infections as well, in part because of its great stature in nucleotide biosynthesis. In this study, a novel one-step synthesis of OxMP is presented. In addition, characterization of OxMP reveals that it is a potent, fully competitive inhibitor of IMPDH with \( K_i = 240 \pm 50 \) nM. OxMP was also observed to inhibit guanosine monophosphate reductase (GMPR) with IC\(_{50} = 210 \pm 40 \) nM.
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List of Abbreviations

AMP: Adenosine monophosphate
AU: Absorbance Units
GMP: Guanosine monophosphate
GMPR: Guanosine monophosphate reductase
$^1$H-NMR: Proton nuclear magnetic resonance
HPLC: High-performance liquid chromatography
IMP: Inosine monophosphate
IMPDH: Inosine monophosphate dehydrogenase
LC-MS: Liquid chromatography-mass spectrometry
mAU: milliabsorbance units
MPA: Mycophenolic acid
MZP: Mizoribine monophosphate
NAD$^+/NADH$: Nicotinamide adenine dinucleotide/NAD reduced form
NADP$^+/NADPH$: Nicotinamide adenine dinucleotide phosphate/NADPH reduced form
nm: nanometer
nM: nanomolar
OxMP: Oxanosine monophosphate
RNS: Reactive nitrogen species
RP-HPLC: Reverse phase high-performance liquid chromatography
S.D.: Standard deviation

ssDNA: Single-stranded DNA

TEAA: Triethylamine acetate

μL: microliter

μM: micromolar

XMP: Xanthosine monophosphate
Introduction

OxMP

Oxanosine is a novel nucleoside with mild antibiotic activity that was discovered in a *Streptomyces* strain in 1981 (Figure 1, Shimada et al. 1981). The first total synthesis of the molecule was completed in 1983 (Yagisawa et al. 1983).

![Oxanosine](image)

*Figure 1. Oxanosine.*

There are several routes to synthesize oxanosine. Suzuki et al. 1996 developed a synthesis of deoxyoxanosine from deoxyguanosine with sodium nitrite, which simulates the damage caused by reactive nitrogen species (RNS) (Figure 2). However, this route has not been reported for oxanosine or OxMP.
Figure 2. Synthesis of deoxyoxanosine.

The proposed mechanism for this reaction is extraordinarily complicated (Figure 3). It involves loss of diatomic nitrogen, carbocation rearrangement, and ring opening and closing (Suzuki et al. 2000, Glaser et al. 1996).

Figure 3. Proposed mechanism of formation of deoxyoxanosine from deoxyguanosine with nitrous acid. Deoxyoxanosine formation involves evolution of diatomic nitrogen, carbocation rearrangement, and ring opening and closing. The R group is a deoxyribose ring. (Figure from Glaser et al. 1996).
Reactive Nitrogen Species

RNS are nitric oxides that can be, but do not have to be, radical species (Wiseman et al. 1996). There are several common sources of human exposure to RNS. For example, sodium nitrite is used as a preservative and dye in cured meats (Kirchner et al. 1991). There is also significant concern that nitrites are present in drinking water. Drinking water can be contaminated by nitrites from sewage systems, composts, and fertilizers (EPA, Vermont). It is important to note that a low level of certain RNS are essential for humans. In fact, humans manufacture low levels of some RNS. Nitric oxide mediates vasodilation of smooth muscle cells and plays an important role in signal transduction (Ignarro et al. 1987, Ignarro et al. 1990). In addition, there is evidence that RNS are used by the immune system to fight microbial infections (Klebanoff 1993).

RNS damage DNA and other biomolecules. Nitrous acid reacts with exocyclic amino groups of ribonucleosides – guanosine is the most reactive of the nucleosides (Shapiro et al. 1968). RNS can cause cross-linking of the DNA strands (Becker-Jr. et al. 1964). Cross-links are comprised of a chemical bridge that connects deoxyguanosine residues of complementary strands at the sequence 5’-CG-3’ (Kirchner et al. 1991, Shapiro et al. 1977) (Figure 4).

**Figure 4.** Structure of cross-linked deoxyguanosine residues upon treatment with nitrous acid. The R groups are deoxyribose rings connected to the phosphodiester backbone (Figure from Shapiro et al. 1977).
In addition to cross-linking, there are other severe chemical modifications that RNS can induce in nucleic acids such as base deamination, strand breaks, oxanosine formation, and generation of abasic sites (Wink et al. 1991, Tamir et al. 1996, Burney et al. 1999). RNS also cross-link proteins to DNA molecules (Chen et al. 2007). Covalent linkages between transcription factors and DNA molecules have devastating effects on gene expression and the integrity of the genome (Oleinick et al. 1987).

As a result of the physical damage that RNS inflict on nucleic acids and proteins, RNS are highly mutagenic. As early as 1958, it was found that treating tobacco mosaic virus with nitrous acid mutates the virus’s genetic material (Gierer et al. 1958). Treating ssDNA phages with nitrous acid mutates guanosine residues into adenosine residues (Tessman et al. 1964). Deoxyoxanosine triphosphate, the triphosphorylated deoxynucleotide analog of OxMP, is misincorporated into nascent DNA strands by DNA polymerase instead of dGTP (Suzuki et al. 1998). These findings suggest that RNS directly lead to mutations in the genetic material (Suzuki et al. 1998). RNS are also hazardous to human health because they lead to the development of cancer and play a central role in inflammation (Wink et al. 1998, Thomson et al. 1995, Oshima et al. 1994, Vliet et al. 1997).

*The biological activity of oxanosine and its metabolites.*

OxMP is a particularly interesting small molecule because this nucleotide is one of the products formed from RNS damage of nucleotides (Chen et al. 2007). While the effects of RNS damage of DNA are well documented, other RNS products could be inhibitors of the enzymes that utilize nucleotides. Since OxMP is structurally very similar
to GMP, there is a possibility that OxMP inhibits enzymes that bind GMP. Aside from GMP synthetase, OxMP could inhibit other GMP-utilizing enzymes such as GMPR. GMPR converts GMP to IMP and ammonia utilizing an NADPH cofactor (Figure 6) (Patton et al. 2011).

Oxanosine is an inhibitor of GMP synthetase competitive with respect to XMP, but the inhibition is not very potent – $K_i = 740 \mu M$ (Yagisawa et al. 1982). Oxanosine is extremely similar in structure to guanosine (Figure 3). The only difference is that N1 in guanosine is replaced by an oxygen atom (Figure 3). Therefore, it is likely that oxanosine gets phosphorylated to OxMP in vivo (Figure 5). Thus, any observed antibiotic activity of oxanosine in vivo might actually be OxMP activity. It is also likely that OxMP gets phosphorylated further to the triphosphate.

**Figure 5. OxMP.**

OxMP is an inhibitor of IMPDH (Uehara et al. 1985). Rat kidney cells transformed with temperature sensitive RAS protein mimic a cancer phenotype at permissive temperatures. When these cells are treated with oxanosine at a permissive temperature, they revert back to having more of the properties of wild type cells. These cells have low levels of GMP (Itoh et al. 1989). In a temperature dependent Rous
sarcoma strain of rat kidney cells (cancer phenotype at permissive temperatures wild type phenotype at nonpermissive temperatures), cell growth was inhibited *in vitro* ten times more effectively at permissive temperatures than at nonpermissive temperatures. In other words, growth was inhibited more severely in the cancer phenotype cells than in the wild type cells. Furthermore, growth inhibition was rescued by guanosine or GMP (Uehara et al. 1985). These observations, and oxanosine’s inhibition of GMP synthetase are in accord with the theory that oxanosine gets phosphorylated to OxMP *in vivo* then inhibits IMPDH. Oxanosine is a product formed from RNS damage of guanosine (Figure 3).

![Figure 6. GMPR catalyzes the conversion of GMP to IMP and ammonia utilizing NADPH.](image)

*Why study IMPDH?*

IMPDH converts IMP to XMP using NAD$^+$ as an oxidizing agent generating an NADH product (Figure 7). NADH absorbs UV light of wavelength 340 nm, and NAD$^+$ does not absorb at that wavelength. This is a convenient spectroscopic probe that can be used to monitor the IMPDH reaction. IMPDH is a particularly impressive enzyme because it catalyzes two types of chemistry in one active site – a dehydrogenase reaction followed by a hydrolysis reaction (Hedstrom 2009).
IMPDH stands astride a decision point of nucleotide biosynthesis. IMPDH commits IMP to be made into GMP as opposed to AMP (Figure 8).

**Figure 7.** Conversion of IMP to XMP by IMPDH.

**Figure 8.** IMPDH catalyzes the reaction that commits IMP to GMP formation. AMP can also be made from IMP (Figure from Nair et al. 2007).

IMPDH is an attractive drug target because of its crucial role in nucleotide biosynthesis. Furthermore, toxicity from purine starvation results more from guanine
nucleotide depletion than adenosine nucleotide depletion (Cohen et al. 1983). This observation suggests that IMPDH is a more promising drug target than the enzymes that commit IMP to be synthesized to AMP. There are several FDA approved inhibitors that target IMPDH. One inhibitor, MPA, is used as an immunosuppressant.

Inhibition of IMPDH is particularly intriguing for treating cancer. IMPDH activity is elevated as much as thirteen fold in rat hepatoma cells in vivo when compared to wild type cells (Jackson et al. 1975). IMPDHs isolated from hepatoma and wild type cells show similar properties indicating that the enzyme is unchanged (Jackson et al. 1975). This observation suggests that the role of IMPDH in cancer results from increased expression of IMPDH rather than a mutation of the enzyme. However, it is important to note that the research from Jackson et al. 1975 was performed before it was known that there are two mammalian isoforms of IMPDH (Carr et al. 1993). Since then, it has been discovered that the Type II is usually the mammalian isoform that is upregulated in cancers (Nagai et al. 1991, Nagai et al. 1992).

MPA stops angiogenesis of tumors in mice (Chong et al. 2006). Even more convincing, RNAi knockdown of IMPDH prevents proliferation of endothelial cells by causing cell cycle arrest (Chong et al. 2006). P53, which among other functions is an anti-proliferative factor, suppresses IMPDH levels (Liu et al. 1998). VX-944, an IMPDH inhibitor, induces apoptosis in human multiple myeloma cell lines (Ishitsuka et al. 2005). A single dose of another IMPDH inhibitor, tiazofurin, caused tumor growth to decrease 85% in vivo (Weber 1983). These results demonstrate that IMPDH promotes cell proliferation, which is essential for cancer. Furthermore, these data demonstrate that inhibitors of IMPDH have great therapeutic potential to treat cancer.
IMPDH is an important antiviral target. At least part of the mode of action of Ribavirin, a drug used to treat respiratory syncytial infection, comes from inhibition of IMPDH. Tiazofurin, shows broad spectrum antiviral activity (Nair et al. 2007).

Although no drugs that target IMPDH have been approved to treat parasitic infections, IMPDH remains a promising drug target for anti-parasitic drugs. Apicomplexans, such as the pathogens that cause malaria and cryptosporidiosis, generally have streamlined purine biosynthesis pathways and completely rely on salvage pathways instead of de novo pathways. Absence of a de novo pathway might make some apicomplexans particularly vulnerable to IMPDH inhibition (Striepen et al 2004). Inhibition of IMPDH is already used to treat viral infections and could be used to treat parasitic infections as well. Therefore, there is great interest in developing inhibitors for IMPDH.

*Kinetics and mechanism of IMPDH*

IMPDH catalyzes two different types of chemistry in one active site. There is a dehydrogenase reaction followed by a hydrolysis reaction. The enzyme occupies an open conformation for the dehydrogenase reaction and a closed conformation for the hydrolysis reaction. A mobile flap descends into the NAD$^+$ binding site after NADH is released. This flap contains the base that catalyzes the hydrolysis reaction. There is substrate inhibition by NAD$^+$ because it can form a non-productive E-XMP*-NAD$^+$ complex that prevents hydrolysis (Hedstrom 1999).

Binding of substrates to IMPDH is random. However, product release is ordered. NADH must dissociate before XMP. This is because NADH must leave so that E-XMP*
can be hydrolyzed (Wang et al. 1997). Hydride transfer is fast. The rate-limiting step of *Cryptosporidium parvum* IMPDH is an amalgamation of NADH release and E-XMP* hydrolysis (Guillen Schlippe et al. 2005, Digits et al. 1999) (Figure 9). However, IMPDHs from different species have different rate-limiting steps.

![Figure 9. Kinetic Scheme of IMPDH. Substrate binding is random, product release is ordered with NADH being released before XMP, hydride transfer is fast, and NADH release and E•XMP hydrolysis are slow.](image)

The catalytic cysteine attacks the IMP substrate forming the covalent intermediate E-XMP*. The hydride is transferred to the Pro-S side of the NAD⁺ cofactor (Xiang et al. 1997). This intermediate is hydrolyzed in the second step of the reaction (Figure 10).

![Figure 10. Mechanism of IMPDH.](image)
There is conformational flexibility in the active site, which allows the nucleophilic cysteine some mobility. This cysteine is free enough to position itself so instead of attacking C2, it can attack C6 of the inhibitor 6-Cl-purine-ribonucleotide (Figure 11). The nucleophilic cysteine and 6-Cl-purine-ribonucleotide forms a stable covalent adduct (Antonino et al. 1994, Markham et al. 1999).

![Diagram](image)

**Figure 11. Inactivation of IMPDH by 6-Cl-purine-ribonucleotide.**

Surprisingly, the base that catalyzes hydrolysis is an arginine residue (Guillen Schlippe et al. 2004, Guillen Schlippe et al. 2005, Min et al. 2008). The kinetic mechanism has been probed from a structural perspective using crystal structures and substrate analogs (Figure 12).
Figure 12. Crystal structures of Tritrichomonas foetus IMPDH to probe IMPDH catalysis (a) E•SO$_4^{2-}$, model for apoenzyme; (b) E•IMP; (c) E•IMP•TAD, model for E•IMP•NAD$^+$; (d) E•RVP•MPA•NAD$^+$; (e) E•MZP•K$^+$, model for E•XMP*; (f) E•XMP; (figure from Hedstrom et al. 2006).

Previous characterization of OxMP inhibition of IMPDH

Uehara et al. 1985 performed kinetic experiments of OxMP with IMPDH from rat kidney cell-free extracts. The double reciprocal plot is shown below (Figure 13). Uehara et al. classify this inhibition as “nearly competitive.” However, this double reciprocal plot is not indicative of competitive, uncompetitive, or mixed inhibition. The lines of different inhibitor concentrations fail to intersect at a common point, which suggests a more complicated mechanism than simple reversible inhibition.
Figure 13. “Nearly Competitive” inhibition by OxMP (Figure from Uehara et al. 1985).

One likely cause of this unseemly double reciprocal plot is that OxMP could exhibit time dependent inhibition. Mechanism-based inhibitors are often time dependent. Bearing this in mind, I hypothesized that OxMP is a mechanism-based inhibitor and proposed a chemical mechanism of action (Figure 14).

Figure 14. Proposed chemical mechanism of OxMP. Enzyme would not be able to hydrolyze the adduct due to the stability of the carboxylate and thiourea moieties.

The carboxylate and thiourea moieties of the product (DEAD ENZYME in Figure 11) should be stable. Had this not been the correct mechanism, I also considered a similar mechanism in which the nucleophilic cysteine attacks C6 instead of C2 of OxMP as observed for 6-Cl-purine-ribonucleotide (Figure 15). In this case, the urea residue of the product would be particularly stable.
Within this study is presented a novel one-step synthesis and purification of OxMP (pure by $^1$H-NMR and HPLC) by reacting GMP with sodium nitrite with 11% yield. In addition, this study rectifies the incorrect characterization by Uehara et al. of OxMP as a “nearly competitive” inhibitor and reports that OxMP is a fully competitive, potent inhibitor of IMPDH with respect to IMP with $K_i = 240 \pm 50$ nM. Finally, within this study is the preliminary characterization of OxMP’s inhibition of GMPR with $IC_{50} = 210 \pm 40$ nM.
Materials and Methods

Materials

GMP, IMP, and NADPH were obtained from Sigma. Sodium nitrite was obtained from Sigma-Aldrich, and NAD\(^+\) was obtained from Roche. Cryptosporidium parvum IMPDH and Escherichia coli GMPR were expressed and purified in the Hedstrom Laboratory.

Synthesis of OxMP

GMP (0.0884 mmol) and sodium nitrite (0.526 mmol) were added to acetate buffer (4 ml, 3 M, pH = 3.7). The flask was closed from the air with a septum. The reaction was stirred for 24 hours in an oil bath at 37 °C.

RP-HPLC analysis

Triethylamine was added to the reaction mixture until pH = 7 (litmus paper). The reaction mixture was filtered through a 0.2 micron filter cellulose acetate filter. The composition of the reaction mixture was assessed using RP-HPLC on a Varian Prostar HPLC. 20 μL of the mixture was injected onto an analytical octa-decyl-bonded silica (C18) Varian HPLC column (250x4.6mmx1/4”). The mobile phase consisted of TEAA (100 mM, pH=7) (Thermo Electron Corporation pH meter) and acetonitrile. HPLC
chromatograms were analyzed at wavelengths of 260 nm and 300 nm. Purification program involved stepwise increases of acetonitrile. The details of the program are found in Table 1.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow Rate (ml/min)</th>
<th>% TEAA</th>
<th>% Acetonitrile</th>
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</thead>
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<td>19.50</td>
<td>1.00</td>
<td>99.5</td>
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</table>

Table 1. Program used for analytical RP-HPLC.

**Preparatory purification of OxMP**

The reaction mixture was filtered with a 0.2 micron cellulose acetate filter, then loaded directly onto RP-HPLC with C18 Varian DYNAMAX HPLC Column
(250x21.4mm) without adjusting the pH. The mobile phase consisted of a gradient of acetonitrile (0.5% to 20%) in TEAA (100 mM, pH = 7). See Table 2 for details.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow Rate (ml/min)</th>
<th>% TEAA</th>
<th>% Acetonitrile</th>
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<tr>
<td>32</td>
<td>1.00</td>
<td>99.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 2. Program used for preparatory RP-HPLC

Fractions containing product were collected and combined in a 1000 ml round bottom flask. The flask was spun by hand in a dry ice/acetone bath to freeze the sample in a thin layer to facilitate lyophilization. Samples were lyophilized overnight. Several more lyophilizations were performed to ensure purity of product by adding 15 ml of Milli-Q H₂O to the round bottom flask containing the lyophilized solid. Once the solid dissolved, it was transferred to smaller scintillation vial and was once more lyophilized. 5 ml Milli-Q H₂O was added to the solid and lyophilized a final time. Product was resuspended in 1 ml D₂O for NMR analysis. After NMR analysis, this sample was lyophilized, and
resuspended in 500 µL Milli-Q H₂O. This sample was lyophilized once more and finally resuspended in 1 ml Milli-Q H₂O. The concentration was determined using the Beer-Lambert Law with the extinction coefficient of oxanosine \( \varepsilon_{260} = 5100 \text{ M}^{-1}\text{cm}^{-1} \) (Suzuki et al. 2000). The product was stored in 5 µL 11 mM aliquots at –80 °C.

**UV trace**

UV profile of OxMP was determined from wavelengths 200 nm to 340 nm using a Cary 100 Bio UV-Visible spectrophotometer. 1 µL Purified OxMP was mixed with 500 µL Milli-Q H₂O in a quartz cuvette.

**LC-MS**

Electrospray minus LC-MS analysis performed by the Krauss Laboratory in the Department of Chemistry at Brandeis University. The program involved a gradient from 1%-10% acetonitrile in formic acid.

**¹H-NMR**

¹H-NMR (400 MHz, D₂O): \( \delta 7.96 \text{ (s, 1H)}, \delta 5.66-5.64 \text{ (d, 1H)}, \delta 4.55 \text{ (s, 1H)}, \delta 4.26 \text{ (s, 1H)}, \delta 4.10 \text{ (s, 1H)}, \delta 3.78 \text{ (s, 2H)} \)

**IMPDH enzyme kinetics**

In this study, *Cryptosporidium parvum* IMPDH was used. The kinetic properties of this enzyme have been previously characterized (Riera et al. 2008). *C. parvum* IMPDH
activity was assessed by monitoring absorbance at a wavelength of 340 nm on a Cary 100 Bio UV-Visible spectrophotometer. Briefly, NAD$^+$ does not absorb at 340 nm but NADH absorbs at that wavelength ($\varepsilon = 6220 \, \text{M}^{-1} \text{cm}^{-1}$). Therefore, as the IMPDH reaction proceeds, there is an overall increase in absorbance at 340 nm.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}} (\text{s}^{-1})$</th>
<th>$K_{\text{m(IMP)}} , (\mu\text{M})$</th>
<th>$K_{\text{m(NAD$^+$)}} , (\mu\text{M})$</th>
<th>$K_{\text{i(NAD$^+$)}} , (\text{mM})$</th>
</tr>
</thead>
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<td>13</td>
<td>140</td>
<td>4.9</td>
</tr>
</tbody>
</table>

*Table 3. Kinetic characterization of C. parvum IMPDH (values from Riera et al. 2008).*

IMPDH assay buffer consisted of 50 mM Tris, 100 mM KCl, 3 mM EDTA, and 1 mM DTT. The Tris/KCl/EDTA component of the assay buffer was stored in a 10X stock. DTT was added fresh each day because it is less stable than the other reagents in the buffer. NAD$^+$ was also mixed fresh each day. Enzyme reactions were performed at 25 °C. Data was fit using the Michaelis-Menten Model:

$$V_0 = V_{\text{max}}[\text{IMP}]/(K_m + [\text{IMP}])$$

**OxMP inactivation quench**

IMPDH (150 μL of 200 nM) in assay buffer was incubated with OxMP (73 μM) at room temperature for ten minutes. 50 μL was removed and added to assay buffer (400 μL) with IMP (1.25 mM) to quench the reaction for a final quench concentration of 1.1 mM IMP. IMPDH (150 of μL 200 nM) in assay buffer was incubated without inhibitor at room temperature for ten minutes. OxMP (73 μM) was added to this sample, which was then incubated at room temperature for another 10 minutes. Quench performed as
described above. IMPDH (150 µL of 200 nM) in assay buffer was incubated without inhibitor for 20 minutes at room temperature then quenched as described above. Enzymatic reactions were initiated by adding NAD\(^+\) (18 µL of 12.5 mM) in Milli-Q H\(_2\)O for a final concentration of 481 µM NAD\(^+\). Final enzyme concentration in each assay was 21 nM. Each sample was performed in duplicate.

**Time of incubation**

IMPDH (400 µL of 200 nM) in assay buffer was incubated with OxMP (220 µM) at room temperature. 50 µL of this mixture was quenched with IMP in assay buffer (400 µL of 1.25 mM) after 0 minutes, 5 minutes, and 10 minutes. IMPDH (50 µL of 200 nM) was incubated with IMP (400 µL of 1.25 mM) in assay buffer for 10 minutes at room temperature. Reactions were initiated by adding NAD\(^+\) (18 µL of 12.5 mM) in Milli-Q H\(_2\)O. Final concentrations of IMP, NAD\(^+\), and enzyme were 1.1 mM, 481 µM, and 21 nM, respectively.

**\(K_i\) determination**

Initial velocity for IMPDH (20 nM) in assay buffer was determined for the following IMP concentrations: 29 µM, 51 µM, 73 µM, 116 µM, 181 µM, and 290 µM. Concentration of NAD\(^+\) was held constant at 450 µM for all trials. This assay was performed for the following OxMP concentrations: 0 µM, 0.8 µM, 1.6 µM, 2.4 µM. Curve fitting, double reciprocal plot construction, and \(K_i\) determination were
accomplished using Sigma Plot 2000 Enzyme Kinetics Module. $K_i$ was fit to the competitive inhibitor model using the following equation:

$$V_o = V_{max} [IMP]/(K_m(1 + [OxMP]/K_i) + [IMP])$$

**GMPR enzyme kinetics**

*Escherichia coli* GMPR activity was assessed by monitoring absorbance at a wavelength of 340 nm using a Cary 100 Bio UV-Visible spectrophotometer. NADPH, the cofactor, absorbs at 340 nm ($\varepsilon = 6220 \text{ M}^{-1}\text{cm}^{-1}$), but the product generated from this cofactor, NADP$^+$ does not absorb at this wavelength. Therefore, as the reaction proceeds, there is an overall decrease in absorbance at 340 nm.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_{m(GMP)}$ (µM)</th>
<th>$K_{m(NADPH)}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> GMPR</td>
<td>0.35 ± 0.01</td>
<td>3.2 ± 0.5</td>
<td>10.1 ± 0.8</td>
</tr>
</tbody>
</table>

**Table 4. Kinetic characterization of *E. coli* GMPR (values from Patton et al. 2011).**

GMPR assay buffer containing 75 mM Tris, 100 mM NaCl, 1 mM EDTA, and 1 mM DTT at pH = 7.8 was used for this assay. GMPR (50 nM) was assayed with GMP (25 µM), NADPH (140 µM), and OxMP (110 µM). A control was performed without OxMP, and both samples were performed in duplicate.

**GMPR IC$_{50}$ determination of OxMP**

IC$_{50}$ was determined using a Cary 100 Bio UV-Visible spectrophotometer. GMPR was incubated with GMP (9.6 µM) and NADPH (140 µM) with OxMP concentrations
varying from 1 nM to 4800 nM. Curve fitting was accomplished using Sigma Plot 2000. The $K_i$ was estimated using the Cheng-Prusoff Equation (Copeland 2005):

$$K_i = \frac{[IC_{50}]}{(1 + \frac{[GMP]}{K_m})}$$
Results

Synthesis, purification, and chemical characterization of OxMP

The synthesis of OxMP was modeled after the synthesis of deoxyoxanosine published by Suzuki et al. 1996. This clever synthesis uses sodium nitrite to mimic RNS damage with 21.5% yield. Guanosine monophosphate was used as the starting material instead of deoxyguanosine to generate OxMP instead of deoxyoxanosine (Figure 16).

![Synthesis of OxMP from GMP](image)

The reaction mixture was analyzed by RP-HPLC using a C18 Varian DYNAMAX HPLC column (250x21.4mm) (Figure 17, Figure 18). OxMP was also purified using RP-HPLC. HPLC traces were monitored at wavelengths of 260 nm and 300 nm. 260 nm was chosen because most nucleotides absorb well at 260 nm, and Suzuki et al. used 260 nm for their chromatography to purify deoxyoxanosine. HPLC traces were also monitored at 300 nm because OxMP absorbs particularly well at 300 nm while the reactants and byproducts do no absorb well at this wavelength.
Figure 17. Analytical RP-HPLC trace at 260 nm of completed reaction mixture. The peak from 7-8 minutes is XMP and byproducts, the peak from 9-10 minutes is GMP, and the peak from 11-12 minutes is OxMP.

Figure 18. Analytical RP-HPLC trace at 300 nm of completed reaction mixture. Peak from 11-12 minutes is OxMP.

LC-MS analysis of OxMP was performed in the Krauss Lab in the Chemistry Department at Brandeis University. LC-MS was used to confirm that the somewhat harsh reaction conditions had not catalyzed hydrolysis of the phosphate of OxMP (Figure 19).
OxMP should be protonated under the LC-MS conditions used. Therefore the exact mass of OxMP is 364.04 Da. Under ES− conditions, the m/z = 363 peak corresponds to OxMP. This LC-MS trace confirmed that the phosphate of OxMP is not hydrolyzed under the synthesis conditions. However, the LC trace and MS trace also revealed a byproduct (m/z = 381) from the reaction or purification that was still present in the sample. This byproduct is 18 Da greater than the expected product. 18 Da is the mass of one water molecule suggesting that OxMP had been hydrolyzed (Figure 20).

![Figure 20. Proposed hydrolysis product of OxMP.](image)
Once the molecule was fully purified, experiments were completed to make sure OxMP does not spontaneously hydrolyze in the assay buffer used (data not shown). $^1$H-NMR was used to further probe this impurity (Figure 21).

![Figure 21. $^1$H-NMR of OxMP in D$_2$O. Each proton peak has another set of peaks associated with it indicating that there was an impurity in this sample.](image)

This problem of the impurity was fixed by changing the workup conditions of the reaction before purification. In the first synthesis, pure triethylamine was added to the reaction mixture (pH = 3.7) to adjust the pH to the conditions of the RP-HPLC mobile phase (pH = 7). When the completed reaction was loaded onto the HPLC without adjusting the pH beforehand, the byproduct disappeared. OxMP used in the following experiments was pure by $^1$H-NMR (Figures 22, 24).
Figure 22. $^1$H-NMR of pure OxMP in D$_2$O. The extra peaks around the downfield peaks have disappeared indicating that the compound is pure. The peaks not accounted for by Figure 23 are solvent peaks.

Figure 23. Structure of OxMP with the hydrogen atoms detected in the $^1$H-NMR spectrum shown. The labels on the hydrogen atoms correspond to the peaks in Figure 22.
Figure 24. Zoom view of the last two peaks furthest downfield of \textsuperscript{1}H-NMR of OxMP to demonstrate purity.

In addition to the peaks described in the Materials and Methods of this study, there were the following peaks: a water peak from the solvent (\(\delta 4.62 - 4.55\)), residual peaks from the TEAA mobile phase – triethylamine (\(\delta 2.97\) and \(\delta 1.06\)) and acetate (\(\delta 2.00\)), and a silicone standard peak (\(\delta 0.23\)).

To further assess purity of the compound, the purified OxMP was analyzed at 260 nm on the analytical RP-HPLC column (Figure 25, Table 5). 260 nm was chosen because it is the wavelength of light that is absorbed by aromatic systems. Almost all possible byproducts in the OxMP reaction mixture would contain aromatic systems and would absorb at 260 nm.
Figure 25. Analytical column RP-HPLC monitored at 260 nm to determine purity.

<table>
<thead>
<tr>
<th>Peak Number</th>
<th>Name</th>
<th>Time (min)</th>
<th>Quantity (% Area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UNKNOWN</td>
<td>8.11</td>
<td>1.25</td>
</tr>
<tr>
<td>2</td>
<td>OxMP</td>
<td>12.01</td>
<td>96.62</td>
</tr>
<tr>
<td>3</td>
<td>UNKNOWN</td>
<td>13.15</td>
<td>1.19</td>
</tr>
<tr>
<td>4</td>
<td>UNKNOWN</td>
<td>13.44</td>
<td>0.02</td>
</tr>
<tr>
<td>5</td>
<td>UNKNOWN</td>
<td>19.11</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Table 5 Integrations of peaks from RP-HPLC trace in Figure 21. These data indicate purity of OxMP of greater than 97%.

Peak 5 (at 19.11 min) is a peak almost always observed in RP-HPLC spectra that corresponds to generic byproducts coming from the column rather than the sample.

Bearing that in mind, these RP-HPLC data indicate that OxMP was prepared with a purity of greater than 97%.
The UV spectrum of OxMP was determined from 200-340 nm (Figure 26).

![UV spectrum of OxMP](image)

**Figure 26.** *UV trace of OxMP from 200-400 nm. The major peaks are at 246 nm and 285 nm.*

The major UV absorbance peaks are at the wavelengths 246 nm and 285 nm. This spectrum is in accord with the UV spectrum published by Suzuki et al. 1997.

OxMP (4.0 mg) was synthesized and purified from GMP (36 mg). Synthesis and purification were completed with 11% yield.

**Time of incubation**

The starting hypothesis of this study was that OxMP is a time dependent mechanism-based inhibitor of IMPDH. If this hypothesis was correct, incubating OxMP with IMPDH in the absence of IMP for a longer time should result in better inhibition. To assess the binding of OxMP to IMPDH, a method to quench the binding of the inhibitor the enzyme was developed. This was necessary so that it would be possible to incubate samples for different amounts of time but assay them at the same time.
Since OxMP is more similar in structure to IMP than NAD$^+$, the quench that was developed was adding IMP to a final concentration of 1.1 mM IMP – more than 10 times greater than the $K_m$ of IMP. One sample of IMPDH was incubated with OxMP for 10 minutes, quenched, then incubated another 10 minutes at room temperature before assaying. Another sample was incubated at room temperature for 10 minutes, then incubated with OxMP at room temperature for another 10 minutes, quenched, then assayed immediately. These samples were equally inhibited by OxMP indicating that the quenching with 1.1 mM IMP stops further binding of OxMP to IMPDH. A sample of IMPDH without OxMP was incubated for 20 minutes at room temperature to verify that the enzyme was not losing significant activity during the 20 minutes at room temperature (Figure 27, Table 6).

![OxMP inactivation quench](image)

**Figure 27.** Quench with 1.1 mM IMP. Since the samples that were held for 10 minutes after quenching (Wait) and the samples assayed immediately after quenching (Assay immediately) have the same activity, the quench is effective. Wait sample offset down 0.04 to facilitate viewing.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Average Slope (AU/min)</th>
<th>Range (AU/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wait</td>
<td>0.0058</td>
<td>0.0046-0.0068</td>
</tr>
<tr>
<td>Assay Immediately</td>
<td>0.0055</td>
<td>0.0049-0.0060</td>
</tr>
<tr>
<td>No OxMP</td>
<td>0.0125</td>
<td>0.0121-0.0133</td>
</tr>
</tbody>
</table>

**Table 6.** The *Wait* and *Assay Immediately* have statistically identical slopes indicating that the quench with 1.1 mM IMP is effective.

To test the hypothesis that OxMP is a time dependent inhibitor, OxMP was incubated with IMPDH in the absence of IMP for 0, 5, and 10 minutes before quenching (Figure 28, Table 7).

**Figure 28.** Time of incubation with inhibitor did not influence potency of inhibition. 5 min and 10 min lines offset 0.05 AU up and 0.055 AU up respectively to facilitate viewing.
<table>
<thead>
<tr>
<th>Sample / time of incubation</th>
<th>Slope (AU/min)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No OxMP</td>
<td>0.0114</td>
<td>0.0009</td>
</tr>
<tr>
<td>0 minute</td>
<td>0.0031</td>
<td>0.0004</td>
</tr>
<tr>
<td>5 minutes</td>
<td>0.0033</td>
<td>0.0004</td>
</tr>
<tr>
<td>10 minutes</td>
<td>0.0029</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

*Table 7. Incubation time of IMPDH with OxMP does not affect the potency of OxMP.*

Increased incubation time of IMPDH with OxMP in the absence of IMP did not result in better inhibition. This finding indicates that OxMP is not a time dependent inhibitor. So, the working hypothesis was revised such that OxMP is a simple reversible inhibitor of IMPDH.

**$K_i$ determination**

Initial rate of the IMPDH reaction was measured at varying concentrations of IMP (constant NAD$^+$) and several different OxMP concentrations to determine the $K_i$ that characterizes its inhibition (Figure 29, Figure 30). An example of the original data is given for the 73 μM IMP point (Figure 31). The data was fit to a fully competitive model.
Figure 29. Plot of initial velocities with respect to IMP concentration. Proceeding from the topmost curve down, the inhibitor concentrations are as follows: 0 µM, 0.8 µM, 1.6 µM, and 2.4 µM.
Figure 30. Double reciprocal plot of 1/rate as a function of 1/[IMP] fitted to fully competitive inhibition. The lines intersecting at the y-intercept indicate that OxMP is a fully competitive inhibitor of IMPDH.
Figure 31. *Time Course of IMPDH at 73 μM IMP. No OxMP and 2.4 μM lines offset 0.005 AU up and 0.01 AU down respectively to facilitate viewing.*

Figure 29 and Figure 30 display the classic patterns of reversible, fully competitive inhibition. The $K_i$ was determined to be 240 ± 50 nM. Table 8 shows the kinetic constants calculated for the *C. parvum* IMPDH. The values of $k_{cat}$ and $K_m$(IMP) are in good agreement with previous reports (Table 3, Riera et al. 2008).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$(IMP) (μM)</th>
<th>$K_i$(OxMP) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp. IMPDH</td>
<td>2.4</td>
<td>18 ± 4</td>
<td>240 ± 50</td>
</tr>
</tbody>
</table>

*Table 8. Kinetic characterization of OxMP with Cp. IMPDH.*

The data was also to fit to noncompetitive and uncompetitive models as well, but it is clear to tell that the inhibition is best described using a competitive model (Figure 32, Figure 33).
Figure 32. Kinetic data fit to an uncompetitive model – this model does not fit the data.
It was unknown whether OxMP inhibits GMPR, so it was necessary to set up a preliminary experiment to determine if OxMP is an inhibitor of GMPR (Figure 34, Table 9).

**GMPR kinetics**

Figure 33. *Kinetic data fit to a noncompetitive model – this model does not fit the data.*
Table 9. OxMP inhibits GMPR.

Samples treated with OxMP have no enzymatic activity demonstrating that OxMP is an inhibitor of GMPR.

GMPR IC₅₀ determination of OxMP

The IC₅₀ of OxMP’s inhibition of GMPR was determined (Figure 35).
The IC$_{50}$ of OxMP inhibiting GMPR was determined to be 210 ± 40 nM. Using the Cheng-Prusoff Equation assuming OxMP is a competitive inhibitor of GMPR competitive with respect to GMP, the $K_i$ of OxMP’s inhibition of GMPR was estimated to be 160 nM.
Discussion

This study presented a novel synthesis of OxMP in 11% yield. The synthesis and purification described here demonstrate that OxMP was prepared completely pure as assessed by $^1$H-NMR. RP-HPLC analysis revealed that OxMP was prepared with greater than 97% purity. Before the workup of the reaction was changed, a byproduct 18 Da greater than the product was observed in the LC trace, MS trace, and the $^1$H-NMR spectrum. 18 Da is the weight of one water molecule. Therefore, it is likely that the byproduct observed was a water molecule hydrolyzing the oxanosine ring (Figure 20). It is reasonable to suggest that the rapid change in pH caused by adding pure triethylamine during the original workup was catalyzing the hydrolysis of OxMP.

It was impossible to change the pH more gradually during workup with a less concentrated base because this generated an unmanageable volume of reaction mixture to process. Once the reaction conditions were changed so there was not a sharp change in pH, hydrolysis was no longer favorable, and the hydrolysis byproduct was not generated.

It is normal to observe solvent peaks and silicon standards even in a pure $^1$H-NMR spectrum. Triethylamine is a particularly hard molecule to remove from purified product. To reduce the amount of triethylamine in the sample, OxMP was washed several times with Milli-Q H$_2$O. The triethylamine should cause no issue in the experiments. For example, in the $K_i$ determination, the largest volume of inhibitor used was 0.1 μL. This
means that for each assay performed there was at least 1 to 5,000 dilution of any residual
triethylamine present in the purified OxMP. Therefore, there was a negligible amount (if any) triethylamine present in the assays described in this study. Acetate peaks are even less concerning because they are such a common small molecule in biological systems.

In this study, IMPDH activity was monitored at varying IMP concentrations to determine the $K_i$. The mechanism of inhibition with respect to NAD$^+$ was not determined because of the complicating feature of NAD$^+$ substrate inhibition. Since the level of NAD$^+$ was kept constant through the $K_i$ determination experiments, substrate inhibition does not affect the fit or the value of the $K_i$ that was calculated.

Incubating IMPDH with OxMP for different amounts of time before adding IMP did not influence the strength of the inhibition. This experiment suggested that the portrait of OxMP as a mechanism-based inhibitor might not be accurate. This result suggested that OxMP might be a simple reversible inhibitor rather than a time dependent mechanism-based inhibitor. In retrospect, it is of course logical that the quench designed was effective. Time of incubation does not affect inhibition for a fast-equilibrating reversible inhibitor.

The incubating for different amounts of time experiment presented in this study demonstrates that OxMP is not a time dependent inhibitor. It was surprising to find that OxMP is not a time dependent inhibitor, because there was evidence at the start of this investigation that suggested that OxMP was a time dependent, mechanism-based inhibitor. There were two plausible chemical mechanisms for OxMP to function as a mechanism-based inhibitor. Furthermore, the homely double reciprocal plot published by Uehara et al. 1985 looked like it was caused by time dependence (Figure 13). The correct
double reciprocal plot was presented in this study (Figure 30). The inaccuracies in the plot presented by Uehara et al. 1985 likely stem from an artifact other than time dependence of the inhibitor. Rather than purified protein as was used in this study, Uehara et al. 1985 assayed the enzymatic activity of IMPDH using a similar assay (monitoring NADH production) in cell-free extracts from rat kidney cells. A cell-free extract system is much more complicated than a purified protein system. There are many other proteins that utilize the reactants and products of the IMPDH reaction that would be present in a cell-free extract. It is possible that other enzymes were either consuming or producing NADH. Since NADH is the spectroscopic probe in this assay, some of the increase in absorbance observed by Uehara et al. 1985 might have been caused by NADH production by enzymes other than IMPDH. It is possible that Uehara et al. 1985 did not see as much activity as they should have because the NADH produced by IMPDH was being consumed by other enzymes. Either of these possibilities would render the Uehara et al. 1985 data meaningless.

My study also evinced that OxMP is a potent, fully competitive inhibitor of IMPDH with respect to IMP. Generally, inhibitors with nanomolar $K_i$ values are considered to be potent inhibitors. The $K_i$ of OxMP is $240 \pm 50$ nM. However, there are more potent inhibitors of the *C. parvum* IMPDH available. For example MZP inhibits this IMPDH with $K_{is} = 11$ nM (Umejiego et al. 2004). Potent inhibitors are intrinsically interesting inhibitors because they can have greater influence in biological systems. OxMP inhibits GMPR with $IC_{50} = 210 \pm 40$ nM. Using the Cheng-Prusoff Equation, the $K_i$ of OxMP’s inhibition of GMPR was predicted to be $K_i = 160$ nM. Future directions
include experimentally determining the $K_i$ that describes OxMP’s inhibition of GMPR as well as characterizing OxMP’s inhibition of IMPDHs from other species.
11. EPA. Basic information about nitrite (measured as nitrogen) in drinking water, in Drinking water contaminants.
http://water.epa.gov/drink/contaminants/basicinformation/nitrite.cfm

References


52. Vermont Department of Health. Nitrates and nitrites in drinking water.


Appendix - Expression and purification of the *Cryptosporidium parvum* thymidylate kinase

In addition to the work on OxMP, I worked on expressing the *Cryptosporidium parvum* thymidylate kinase (TMPK). TMPK catalyzes the conversion of thymidylate monophosphate (TMP) to thymidylate diphosphate (TDP) (Figure A1). This enzyme uses an ATP cofactor.

![Figure A1](image.png)

**Figure A1.** TMPK catalyzes the conversion of TMP to TDP using ATP as a cofactor.

The *C. parvum* TMPK is an appealing drug target for several reasons. First, there have been no effective drugs developed to treat cryptosporidiosis. Second, there are significant structural differences between the *C. parvum* TMPK and the human TMPK. Only 38% of the amino acids found in the human TMPK are conserved in the *C. parvum* TMPK. Specifically, there is 60% conservation in the TMP binding site and only 24% conservation in the AMP binding site (Figure A2).
Figure A2. Crystal structure of human TMPK. Red residues are conserved in the C. parvum homolog, blue residues are not conserved. Figure constructed using Chimera.

*C. parvum* TMPK was cloned from *C. parvum* genetic material. This enzyme was placed under control of an IPTG-inducible promoter. pET28a, the plasmid that *C. parvum* TMPK was cloned into also contained a thrombin-cleavable N-terminal hexahistidine tag to be used for purification.

Parasitic enzymes are known to be hard to express. However successful expression conditions were determined for a 5 ml expression test. Expression was carried out in Tuner Cells (Lac permease knockouts to reduce the amount of IPTG that enters cells). Tuner cells were induced with 0.025 mM IPTG at an OD of 2.4. Cells were incubated after induction for 24 hours at 16 °C (Figure A3, Figure A4).
Figure A3. Expression of C. parvum TMPK in Tuner cells induced with 0.025 mM IPTG at OD 2.4 for 24 hours at 16 °C. SDS-PAGE gel stained with Coomassie. Note the thicker band at 20 kDa in the lysate of the 0.025 mM IMPTG lane compared to the lysate of the uninduced lane. Also take note that much of the protein express ended up in the pellet.
Figure A4. SDS-PAGE gel of expression stained with chemical that recognizes hexahistidine tags. There is a thicker band at 20 kDa in the induced lysate compared to the uninduced lysate.

Unfortunately these 5 ml expression test tests did not scale well to a volume that generated enough enzyme to characterize. These expression conditions could be used as a starting point to try and analyze express, purify, and characterize the *C. parvum* TMPK.