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Inhibition of Inosine Monophosphate Dehydrogenase (IMPDH) by the Antiviral Compound, 2-Vinylinosine Monophosphate

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Abstract—A new enzyme-mediated synthesis of 2-vinylinosine, a compound with broad-spectrum RNA antiviral activity, is described. In order to understand the mechanism of action of this compound, we synthesized its monophosphate and investigated the behavior of that compound toward the enzyme, inosine monophosphate dehydrogenase (IMPDH), a key enzyme involved in the biosynthesis of nucleotides. 2-Vinylinosine monophosphate is a potent inhibitor of IMPDH with a K_i of 3.98 μM ($k_{inact} = 2.94 \times 10^{-2}$ s^{-1}). The antiviral activity of 2-vinylinosine may be explained by its cellular conversion to the monophosphate through the sequential action of PNP and HGPRT and subsequent inhibition of IMPDH by the cellularly produced 2-vinylinosine 5′-monophosphate.
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Introduction

Inosine 5′-monophosphate dehydrogenase (IMPDH; EC 1.2.1.205) catalyzes the NAD^+-dependent oxidation of inosine 5′-monophosphate (IMP) to xanthine 5′-monophosphate (XMP). Its role at the metabolic branch point in the de novo purine nucleotide biosynthetic pathway makes it a useful target in the development of drugs for antiviral and anticancer chemotherapy and in the immunosupressant area.[1–8] The human enzyme exists in two isoforms: Type I which is expressed in normal cells and Type II which predominates in neoplastic and fast replicating cells.[1,3,4,9] The activity of the enzyme increases markedly in rapidly proliferating cells such as leukemic cells and hepatoma cells. Thus, inhibitors of IMPDH have been suggested as being of considerable interest in cancer chemotherapy.[4,7,8] The mechanism of action of IMPDH involves interaction of the enzyme and coenzyme (NAD^+) complex at the 2-position of IMP, which is of significance in the design of inhibitors of the enzyme.[10–12] It has been shown that IMPDH contains a cysteine residue at the active site that attacks the 2-position of substrate IMP and forms a covalent complex (E-IMP). This intermediate (E-IMP) forms XMP by transferring hydride to NAD^+ and then undergoing hydration at the 2-position which is followed by ejection of the enzyme. IMP, the natural substrate of IMPDH, appears to protect the active site from inactivation by inhibitors.[11] X-ray crystallographic data suggest that a covalent adduct may be formed between Cys-331 of IMPDH and IMP during catalysis.[13]

Several compounds with antiviral activity have been found to be inhibitors of IMPDH. For example, ribavirin, a competitive inhibitor of IMPDH,[14] has broad-spectrum antiviral activity against DNA and RNA viruses.[15] Ribavirin has been approved as an inhaled antiviral agent for the treatment of respiratory syncytial infection and, orally in combination with alpha interferon (IFN-α), for the treatment of chronic hepatitis C virus (HCV) infection.[15–17] 3-Deazaguanosine monophosphate, also a competitive inhibitor of IMPDH,[16] has been discovered to be inhibitory towards several RNA viruses.[18] 5-Ethynyl-1-β-D-ribo-furanosylimidazole-4-carboxamide or EICAR, another inhibitor of IMPDH as its MP, is a potential antiviral and antileukemic agent.[19,20] This paper reports on an enzyme-mediated synthesis of 2-vinylinosine monophosphate (2-VIMP) and a comprehensive study of its inhibition of the enzyme, IMPDH. 2-Vinylinosine monophosphate appears to be the cellularly active form of 2-vinylinosine, a compound discovered by us and found to have broad-spectrum RNA antiviral activity.[21]

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Results

Materials

IMP, NAD^+, and DTT were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *Escherichia coli* cells were grown at the University of Iowa Biocatalysis and Bioprocessing Center (Iowa City, IA, USA). Green A-Sepharose gel was procured from Millipore (Bedford, MA, USA). All other chemicals used were of analytical grade.

Purification of IMPDH

The enzyme was purified from *E. coli* B3 cells using a modification of the procedure previously described.[22] Bacterial cells (80 g) were harvested and disrupted (French Press) in 200 mL of 50 mM Tris–HCl (pH 8.0), 0.15 M KCl, 2 mM 2-mercaptoethanol, 1 mM EDTA and 0.1 mM PMSF (buffer A). The resulting solution was centrifuged at 7000 rpm in order to remove cell debris. The supernatant was collected and saturated with solid ammonium sulfate and the resulting solution was centrifuged at 20,000 rpm for 30 min. The pellet was collected, dissolved in buffer A and dialyzed first against buffer A and then against buffer B (20 mM Tris–HCl buffer pH 8.0 containing 0.15 M KCl, 0.1 mM EDTA and 0.8 M urea). The pellet, which contained IMPDH activity, was further purified by loading on a Green A-Sepharose (Matrix Green Resin, Millipore) column. The column was developed in buffer B. The bound enzyme was eluted with 0.15–1.0 M KCl linear gradient in buffer B. The fractions with IMPDH activity were concentrated and dialyzed against 50 mM Tris–HCl (pH 8.0), 0.1 M KCl and 1 mM EDTA. A total of 80 μmol/ min activity units were recovered.

Assay procedure for IMP dehydrogenase

The assays were typically performed with appropriate amounts of enzyme in 50 mM Tris–HCl buffer pH 8.0 containing 0.15 M KCl, 1 mM DTT and 1 mM EDTA using substrate concentrations of 0.5 mM IMP and 1.5 mM NAD^+ at 25 C. The production of NADH was monitored by the change in absorbance at 340 nm using a Cary 3 UV spectrophotometer. One unit of enzyme has been defined as the amount of enzyme that produces 1 μmol of NADH per min at 25 °C under the assay conditions.

Kinetics of IMPDH inhibition by 2-vinylinosine monophosphate (2-VIMP)

Purified IMPDH was incubated with various concentrations of 2-VIMP at 25 °C. After various intervals of time, the reaction was started by addition of the substrate (IMP) and the enzymatic activity was determined by measuring the absorbance at 340 nm. During the data collection less than 10% of the substrate was consumed.

Another experiment of inhibition was also performed separately where the enzyme was incubated with inhibitor in presence of substrate (IMP) for various time intervals to determine the extent of protection of the enzyme by the substrate.

Inactivation, denaturation and renaturation of *E. coli* IMPDH

E. coli IMPDH was incubated with 25 μM of 2-VIMP for 4 h at 25 °C. After this, urea was added to a final concentration of 8 M and the resulting solution was incubated for another 1 h at this temperature. The denatured enzyme was dialyzed overnight against the assay buffer [50 mM Tris–HCl (pH 8.0), 0.15 M KCl, 1 mM EDTA containing 1 mM DTT] with 3–4 intermittent changes of buffer. A control sample of *E. coli* IMPDH (in the absence of inhibitor 2-VIMP) was treated likewise following the same procedure. Enzymatic activity for both control and treated enzyme was monitored at each step (Fig. 1).

Synthesis of 2-vinylinosine and its 5-monophosphate. 6-Chloro-2-vinyl-9-(β-D-ribofuranosyl)purine (**1**) was synthesized from 6-chloro-2-iodopurine ribonucleoside triacetate by palladium-mediated coupling [with $Pd(CH_3CN)_2Cl_2$, tributylvinyltin, DMF, 90–95 °C] followed by careful deprotection of the acetate groups with methanolic ammonia. The general procedure for the coupling reaction has been previously described by us.[23] The product was purified by HPLC on a C_{18} column and eluted with 35% MeOH/H_2O: mp 201–202 C (decomp); UV (MeOH) λ_{max} 277 (ε 11,614), 228 (ε 17,631); ^1H NMR (CD_3OD) δ 3.86 (m, 1H), 3.90 (m, 1H), 4.15 (m, 1H), 4.38 (m, 1H), 4.72 (m, 1H), 5.70 (dd, $J = 1.4$, 10.2 Hz, 1H), 6.11 (d, $J = 5.3$ Hz, 1H), 6.64 (m, 1H), 6.81 (m, 1H), 8.73 (s, 1H); ^{13}C NMR (CD_3OD) δ 62.8, 72.0, 75.8, 87.4, 90.8, 125.1, 131.4, 136.1, 147.0, 151.3, 153.1, 160.1.

2-Vinyl-9-(β-D-ribofuranosyl)hypoxanthine (2). To a solution of compound **1** (20 mg) in 0.1 M potassium phosphate buffer pH 7.0 (3.0 mL) was added adenosine deaminase (calf spleen, 250 units). The reaction mixture was left for 4 days at 25 °C. Progress of reaction was followed by monitoring the bathochromic shift of the

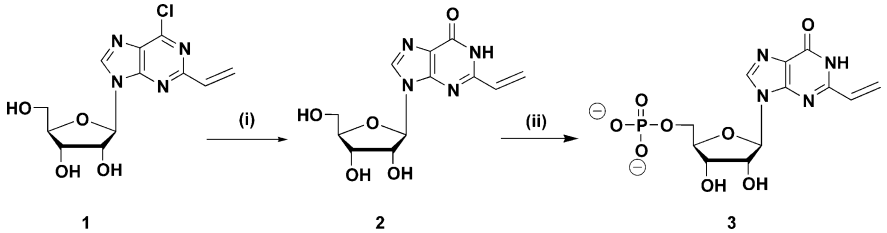

Figure 1. Chemoenzymatic synthesis of IMPDH inhibitor, 2-vinylinosine monophosphate (2-VIMP). Reagents: (i) adenosine deaminase, 0.1 M phosphate buffer; (ii) $POCl_3$, triethyl phosphate.

λ_{max} at 277 nm to a final value of about 300 nm. The enzyme was filtered using an Amicon Ultra Filtration device with YM-10 membrane and the solution was purified by HPLC on a C_{18} column with water/methanol. Pure product was eluted with 30% methanol/water to give 13 mg (70% yield) of compound **2:**[23] mp 225–230 °C (decomp); UV (MeOH) λ_{max} 300 (ε 6286), 256 (ε 6345); ^{1}H NMR (CD_3OD) δ 3.30 (m, 1H), 3.83 (m, 1H), 4.11 (m, 1H), 4.34 (m, 1H), 4.65 (m, 1H), 5.83 (m, 1H), 6.03 (d, J = 5.5 Hz, 1H), 6.57 (m, 2H), 8.30 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 62.9, 72.0, 75.9, 87.3, 89.6, 124.7, 126.5, 130.8, 141.1, 150.2, 153.6, 158.7.

2-Vinyl-9-(β-D-ribofuranosyl) hypoxanthine 5′-monophosphate (3). To a mixture of compound **2** (52 mg, 0.17 mmol) and anhydrous triethyl phosphate (0.03 mL) at 0 °C was added anhydrous $POCl_3$ (0.1 mL).[24] The reaction mixture was stirred at 0 °C for 6 h and neutralized with $NaHCO_3$. Ether was added and the resulting precipitate was separated by centrifugation. The white precipitate was dissolved in water and was purified by HPLC on a C_{18} column with water–methanol. Pure product was eluted with 100% water. Removal of the solvents under reduced pressure afforded compound (**3**) (28 mg) in 43% yield: mp 228–230 °C (decomp); UV (H_2O) λ_{max} 290 (ε 4937), 256 (ε 7826); ^{1}H NMR (CD_3OD) δ 3.94 (m, 3H), 4.24 (m, 1H), 4.40 (m, 1H), 5.82 (m, 1H), 6.04 (d, J = 5.8 Hz, 1H), 6.42 (m, 1H), 6.52 (m, 1H), 8.34 (s, 1H); ^{13}C NMR (D_2O) δ 65.2, 71.7, 75.6, 85.4, 88.3, 123.5, 127.7, 129.6, 141.1, 150.7, 154.2, 159.6; ^{31}P NMR (D_2O): δ 1.16.

Discussion

Chemoenzymatic synthesis of 2-vinylinosine and its monophosphate

Our new synthesis of 2-vinylinosine (**2**) from the hydrolytic dechlorination of 6-chloro-2-vinyl-9-(β-D-ribofuranosyl) purine (**1**) with adenosine deaminase represents an excellent approach to the synthesis of this and related compounds through this chemoenzymatic approach. Compound **2** was phosphorylated using standard phosphorylation methodology and the monophosphate was purified by HPLC on a C_{18} column.

Kinetics of inhibition/inactivation of IMPDH

The inactivation/inhibition of IMPDH followed psuedo first-order kinetics as demonstrated by the exponential loss of activity (Fig. 2). The data were analyzed using the following equation:

$$\ln(V_t/V_0) = -k_{obs}t$$

where V_t is the activity at time t and V_0 is the activity at time $t = 0$. The replots of apparent rate constants (k_{obs}) versus inhibitor (2-VIMP) concentration displayed biphasic hyperbolic behavior (Fig. 3). This behavior indicates that the inhibition/inactivation caused by 2-VIMP on IMPDH follows a two-step mechanism:

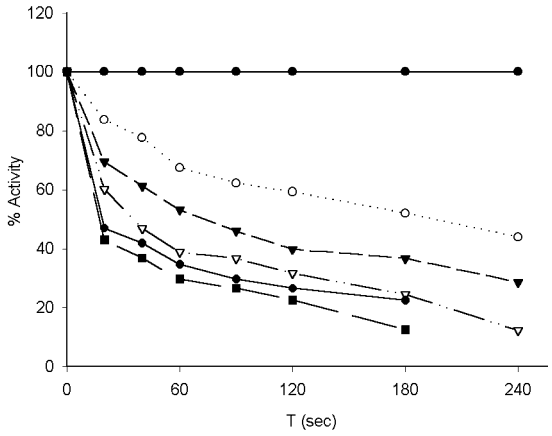

Figure 2. Inhibition of *E. coli* IMPDH by 2-VIMP. Enzyme assay: IMP = 0.5 mM, NAD+ = 2.5 mM, (●) no inhibitor, horizontal line, 2-VIMP = (○) 0.71 μM, (▼) 1.0 μM, (▽) 1.67 μM, (●) 3.5 μM, (■) 5.0 μM.

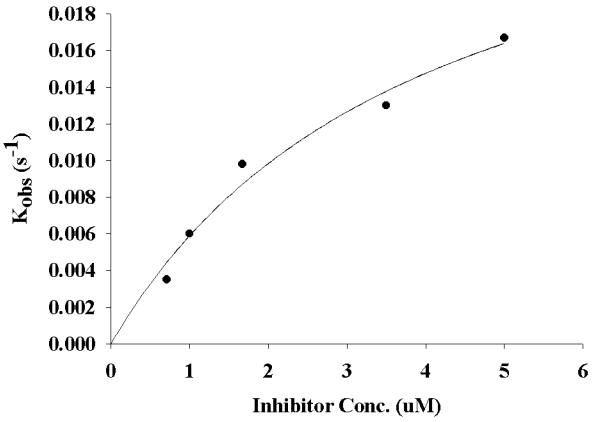

Figure 3. Plot of apparent rate constant (k_{obs}) versus inhibitor (2-VIMP) concentration.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_{inact}} E - I$$

where $E{\cdot}I$ is a reversible complex and $E-I$ is the irreversibly inactivated enzyme. Support for this type of mechanism comes from the work on inactivation of *E. coli* and human type-2 IMPDHs by 6-chloropurine ribonucleoside monophosphate.[25]

The values of k_{obs} for VIMP were then fitted into the following equation:

$$k_{obs} = \frac{k_{inact}[I]}{K_i + [I]}$$

where [I] is the inhibitor concentration, k_{inact} is the rate constant for inactivation and K_i is the apparent dissociation constant of E·I. The values of k_{inact} and K_i were then calculated by plotting $1/k_{obs}$ versus $1/[I]$ values (Fig. 4).

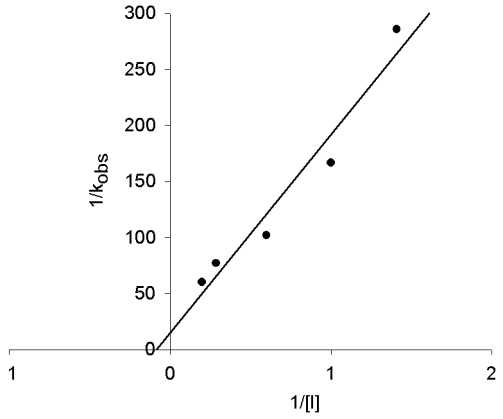

Figure 4. Plot of $1/K_{obs}$ versus $1/[I]$ for calculation of k_{inact} and K_i.

The data thus obtained show that 2-vinylinosine monophosphate is a potent inhibitor of IMPDH with a K_i of 3.98 μM and a k_{inact} of 2.94×10^{-2} s^{-1}. A side-by-side comparison with the well-known IMPDH inhibitor, 6-chloropurine ribonucleoside monophosphate, gave values for K_i and k_{inact} indicative of a weaker inhibitor ($K_i = 62.0$ μM and $k_{inact} = 7.60\times10^{-2}$ s^{-1}).

Attempts made to recover the activity of VIMP-inactivated enzyme were unsuccessful. On the other hand, a control experiment of urea denaturation followed by renaturation, showed almost 80% recovery of activity. When IMPDH was incubated with various concentrations of inhibitor, 2-VIMP, in the presence of excess of its normal substrate, IMP, the enzyme inhibition/inactivation again followed psuedo first-order kinetics, and the data were as follows: $K_i = 163.0$ μM and $k_{inact} = 3.30\times10^{-2}$ s^{-1}. These experiments suggest that the binding of IMPDH with 2-VIMP may involve a reversible competitive step which is followed by the inactivation process. The results indicate that the inhibition caused by 2-VIMP may be due to the formation of an apparently irreversible covalent bond between the inhibitor and IMPDH where the vinyl group conjugated to the purine ring of VIMP acts as a Michael acceptor presumably for Cys-331 of the enzyme.

2-Vinylinosine is not a substrate for adenosine kinase but it is a cleaved slowly by purine nucleoside phosphorylase (PNP, calf spleen, 30% cleavage)[26] and the resulting base appears to be a substrate for hypoxanthine–guanine phosphoribosyl transferase (HGPRT) (10% conversion).[27]

Conclusion

2-Vinylinosine has been found to possess broad-spectrum antiviral activity against a number of exotic RNA viruses including JEV, PIC, PT, VEE and YF.[21] The mechanism of this antiviral activity may be associated with the ability of the cellularly produced monophosphate of this compound to be an inhibitor of IMPDH. Preliminary results from our laboratory suggest that its cellular conversion to the monophosphate may be through the sequential action of PNP and HGPRT.

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