A Potent, Covalent Inhibitor of Orotidine 5′-Monophosphate Decarboxylase with Antimalarial Activity

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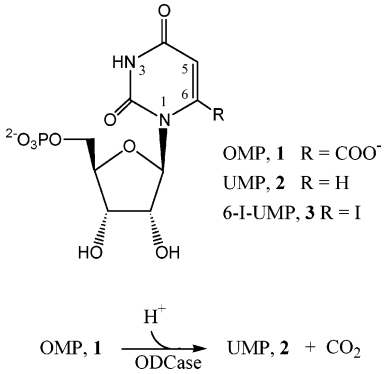
Orotidine 5′-monophosphate decarboxylase (ODCase) has evolved to catalyze the decarboxylation of orotidine 5′-monophosphate without any covalent intermediates. Active site residues in ODCase are involved in an extensive hydrogen-bonding network. We discovered that 6-iodouridine 5′-monophosphate (6-iodo-UMP) irreversibly inhibits the catalytic activities of ODCases from *Methanobacterium thermoautotrophicum* and *Plasmodium falciparum*. Mass spectral analysis of the enzyme−inhibitor complex confirms covalent attachment of the inhibitor to ODCase accompanied by the loss of two protons and the iodo moiety. The X-ray crystal structure (1.6 Å resolution) of the complex of the inhibitor and ODCase clearly shows the covalent bond formation with the active site Lys-42 residue. 6-Iodo-UMP inhibits ODCase in a time- and concentration-dependent fashion. 6-Iodouridine, the nucleoside form of 6-iodo-UMP, exhibited potent antiplasmodial activity, with IC_{50}s of 4.4 ± 1.3 μM and 6.2 ± 0.7 μM against *P. falciparum* ItG and 3D7 isolates, respectively. 6-Iodouridine 5′-monophosphate is a novel covalent inhibitor of ODCase, and its nucleoside analogue paves the way to a new class of inhibitors against malaria.

Introduction

Orotidine 5′-monophosphate decarboxylase (ODCase)[a] is one of the most proficient members of the enzymic world. This enzyme, present in bacteria, archaea, parasites, and higher species including mammals, catalyzes the decarboxylation of orotidine monophosphate (OMP, **1**) to uridine monophosphate (UMP, **2**, Scheme 1).[1,2,3] This enzyme is particularly noteworthy because it exhibits an extraordinary rate enhancement of over 17 orders of magnitude compared to the uncatalyzed reaction in water at pH 7.0 and 25 °C.[4] Most of the other decarboxylases in Nature use either a cofactor or covalent intermediates during the process of decarboxylation.[5,6] For example, thiamine diphosphate-dependent indolepyruvate decarboxylase (IPDC) uses thiamine as a cofactor, and there are covalent intermediates formed with the cofactor during the decarboxylation process. In fact, ODCase is thought to be quite unusual in catalyzing decarboxylation with such proficiency without the help of any cofactors, metals, or covalent-intermediates.[1] ODCase plays a central role in the *de novo* synthesis of UMP, which in turn forms the basis for the synthesis of other pyrimidine nucleotides such as UTP, TTP, and CTP. Thus, the inhibition of ODCase activity could have potential in the treatment of a variety of diseases due to its central role in nucleotide synthesis.

Scheme 1



In the past two decades, several groups have investigated various inhibitors of ODCase and their potential in drug development. For example, *Plasmodia* species such as *P. falciparum* and *P. vivax* are dependent on the *de novo* synthesis for pyrimidine nucleotides due to the absence of the salvage pathway.[7] In humans, however, pyrimidines are obtained from both the *de novo* and salvage pathways.[8] Thus, inhibition of malarial ODCase has been proposed as a strategy to design compounds directed against malaria, and several analogues of orotate have been investigated as potential drugs against the malaria parasite.[9−11] Pyrazofurin, 6-azauridine 5′-monophosphate, xanthosine 5′-monophosphate (XMP), 6-thiocarboxami-

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[a] Abbreviations: ODCase, orotidine 5′-monophosphate decarboxylase; UMP, uridine 5′-monophosphate; OMP, orotidine 5′-monophosphate.

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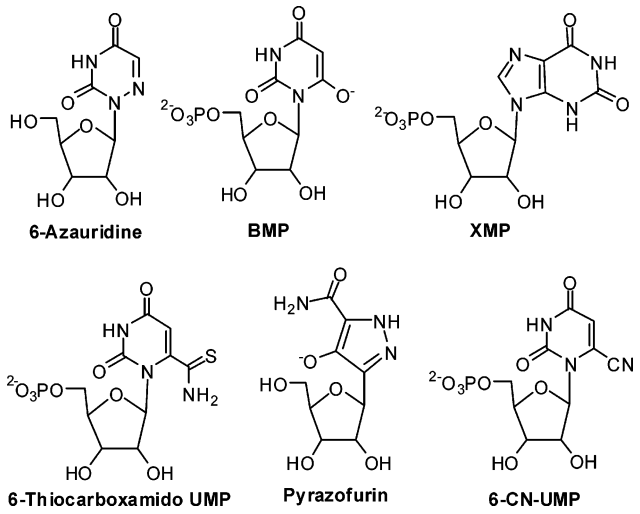
Chart 1. Structures of ODCase Inhibitors



douridine 5′-monophosphate, and barbituric acid ribonucleoside 5′-monophosphate (BMP) were some of the potent inhibitors that were studied against ODCase (Chart 1).[10,12] In general, most of the studies targeting ODCase focused on malaria and cancer; however, the development of inhibitor candidates has been limited due to their toxicities and lack of specificity.[10]

Aside from its obvious pharmacological interest, ODCase has been a favorite enzyme for biochemists and structural biologists due to its unusual catalytic properties. A number of mechanisms were proposed prior to and after the X-ray crystal structures for several ODCases became available in 2000.[13−16] Although ideas of covalent catalysis were briefly discussed, none of the mechanisms presented included covalent species formation as a key step during the decarboxylation by ODCase. An analysis of the catalytic site of ODCase revealed two aspartate residues (Asp70 and Asp75B, the latter contributed by the second subunit of the dimeric ODCase) and two lysine residues (Lys42 and Lys72) that are held via a strong network of hydrogen bonds (Figure 1). Analyses of several cocrystal structures of ODCase with a variety of ligands confirm that these residues are held tightly in their respective positions in the active site and there is less than 0.5 Å movement in the positions of the side chains of these residues. Existing evidence does not support any active site residue forming a covalent bond to either the substrate during catalysis or to any known inhibitor.[1,14,17−19]

Our group recently reported that ODCase could facilitate biochemical reactions other than decarboxylation. We investigated 6-cyanouridine 5′-monophosphate (Chart 1) as a potential bioisosteric inhibitor of ODCase using X-ray crystallography and enzymology.[21,22] ODCase catalyzes the surprising conversion of the chemically stable 6-cyanouridine 5′-monophosphate into BMP, albeit slowly with a half-life of 5 h, in what can be categorized as a "pseudo-hydrolysis" process.[20] Even in this unusual enzymatic conversion, we did not observe any covalent interactions between residues in the catalytic site of wild-type ODCase and the substrate or the product.[20,21] 6-Cyanouridine 5′-monophosphate also exhibited noncovalent, competitive inhibition of ODCase activity with a K_i of 29 ± 2 μM.[21] Thus far, neither enzymatic nor structural studies on ODCase produced any evidence that the enzyme might use the "catalytically" important residues for anything but noncovalent catalytic function.

As part of our continuing investigations on ODCase, we synthesized and investigated 6-iodouridine 5′-monophosphate (**3**) as a potential inhibitor of ODCase.[22,23] We discovered that

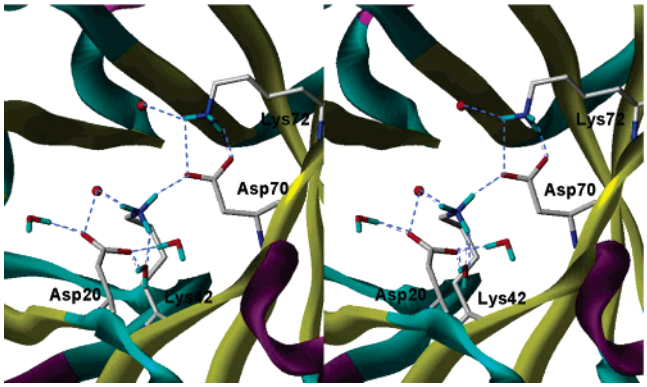


Figure 1. Stereo representation (crossed-eye) of the hydrogen-bonding network in the active site of *Mt* ODCase (PDB code: 1DV7). Key residues Lys72, Asp70, Lys42, and Asp20 are labeled; several crystallographic waters are also shown as spheres in red.

a covalent bond is formed between one of the enzyme active site residues and the pyrimidine ring of compound **3**. Additionally, its nucleoside form, 6-iodouridine (**7**), exhibited potent antiparasitic activities against *P. falciparum*. Here, we reveal the first inhibitor of ODCase that leaves the established catalytic path and leads to a covalently attached product complex. We report the structural and enzymatic evidence on ODCase inhibition (*M. thermoautotrophicum* and *P. falciparum*) and the antiplasmodial activities of this novel inhibitor.

Experimental Section

General. All reactions were performed under a nitrogen atmosphere. All solvents and reagents were obtained from commercial sources. Column chromatography purifications were performed using silica gel (60 Å, 70−230 mesh). The NMR spectra were recorded on a Varian spectrometer (300 and 400 MHz for ^{1}H, 75 and 100 MHz for ^{13}C, and 121.46 MHz for ^{31}P). The chemical shifts are reported in δ ppm using tetramethylsilane as the reference for the ^{1}H NMR spectra, and phosphoric acid as an external standard for the ^{31}P spectrum. Mass spectra for the inhibitors were obtained in a Q-Star mass spectrometer using ESI or EI technique. All enzyme assays were performed at 55 °C or 37 °C using a VP-ITC microcalorimeter (MicroCal, Northampton, MA) according to previously published procedures.[21] The pH of the buffers was measured with a Corning 430 pH meter. Mass spectra for the enzyme and the complex were obtained in a Q-TOF Mass Spectrometer (Waters Micromass, Manchester, UK) at the Mass Spectrometry Facility, Advanced Protein Technology Centre, The Hospital for Sick Children, Toronto, Canada and MassLynx software was used for data analysis.

Synthesis. 5′-*O*-(*tert*-Butyldimethylsilyl)-2′,3′-*O*-isopropylideneuridine (5). A stirred suspension of uridine **4** (1 g, 4.1 mmol) in anhydrous acetone (50 mL) was treated with H_2SO_4 (0.5 mL) dropwise at room temperature, and the resulting mixture was stirred for an additional 1 h. The reaction was then neutralized with Et_3N and was concentrated. The crude mixture was purified by column chromatography (5−8% MeOH:$CHCl_3$) to afford 2′,3′-*O*-isopropylideneuridine (1.15 g, quant.) as a white solid. ^{1}H NMR ($CDCl_3$) δ 1.36 (s, 3H, CH_3), 1.57 (s, 3H, CH_3), 3.80 (dd, 1H, H-5′), 3.91 (dd, 1H, H-5′′), 4.26−4.30 (m, 1H, H-4′), 4.95 (dd, 1H, H-3′), 5.02 (dd, 1H, H-2′) 5.56 (d, 1H, H-1′), 5.72 (d, 1H, H-5), 7.36 (d, 1H, H-6).

A stirred solution of 2′,3′-*O*-isopropylideneuridine (0.2 g, 0.7 mmol) in anhydrous CH_2Cl_2 (3 mL) was treated with imidazole (0.095 g, 1.4 mmol) and TBDMSCl (0.105 g, 0.7 mmol) at 0 °C. The reaction mixture was brought to room temperature and

stirred for an additional 1 h. The solvent was evaporated under vacuum, and the crude material was dissolved in ethyl acetate (30 mL), washed with water (15 mL) and brine (15 mL), and dried (Na_2SO_4). Evaporation of the solvent and purification of the crude by column chromatography (5% MeOH in $CHCl_3$) yielded compound **5** (0.27 mg, 96% yield) as a foam: ^{1}H NMR ($CDCl_3$) δ 0.10 (s, 6H, CH_3), 0.90 (s, 9H, CH_3), 1.36 (s, 3H, CH_3) 1.59 (s, 3H, CH_3), 3.79 (dd, 1H, H-5′), 3.92 (dd, 1H, H-5″), 4.30−4.33 (m, 1H, H-4′), 4.67 (dd, 1H, H-3′), 4.75 (dd, 1H, H-2′), 5.66 (d, 1H, H-5), 5.96 (d, 1H, H-1′), 7.68 (d, 1H, H-6), 8.47 (brs, 1H, -NH).

5′-*O*-(*tert*-Butyldimethylsilyl)-6-iodo-2′,3′-*O*-isopropylideneuridine (6). A stirred solution of LDA (0.62 mL, 1.3 mmol, 2.0 M solution in THF) in anhydrous THF (2 mL) was treated with compound **5** (0.25 g, 0.6 mmol) dissolved in 1.5 mL of anhydrous THF, at −78 °C. After stirring for 1 h, iodine (0.16 g, 0.6 mmol) in anhydrous THF (2 mL) was added and the mixture was stirred for an additional 5 h at the same temperature. The reaction was quenched with AcOH (0.3 mL) and then brought to room temperature and dissolved in ethyl acetate (25 mL). The organic layer was washed with saturated $NaHCO_3$ solution (10 mL), 5% $Na_2S_2O_3$ solution (10 mL), and brine (10 mL) and dried (Na_2SO_4). Evaporation of the solvent and purification of the crude by column chromatography (hexanes−ethyl acetate, 70:30) gave **6** (0.224 g, 68%) as a yellow foam: ^{1}H NMR ($CDCl_3$) δ 0.06 (s, 6H, CH_3), 0.89 (s, 9H, $3CH_3$), 1.35 (s, 3H, CH_3) 1.56 (s, 3H, CH_3), 3.76−3.86 (m, 2H, H5′, H-5″), 4.15−4.20 (m, 1H, H-4′), 4.81 (dd, 1H, J = 4.2, 6.3 Hz, H-3′), 5.18 (dd, 1H, J = 2.0, 6.3 Hz, H-2′), 6.09 (d, 1H, J = 2.0 Hz, H-1′), 6.45 (s, 1H, H-5), 8.78 (brs, 1H, NH).

6-Iodouridine (7). A stirred suspension of compound **6** (0.300 g, 0.572 mmol) in water (2 mL) was treated with 50% aqueous TFA (3 mL) at 0 °C, brought to room temperature, and stirred for 2 h in the dark. Evaporation of the solvent and purification of the crude by column chromatography (10−15% EtOH in $CHCl_3$) afforded compound **7** (0.182 g, 0.49 mmol, 86%) as a light brown solid. UV (H_2O): λ_{max} = 268 nm (ϵ = 8975); ^{1}H NMR (D_2O) δ 3.77 (dd, 1H, H-5′), 3.91 (dd, 1H, H-5″), 3.978−4.032 (m, 1H, H-4′), 4.43 (t, 1H, H-3′), 4.84 (dd, 1H, H-2′), 6.06 (d, 1H, H-1′), 6.67 (s, 1H, H-5). HRMS (ESI) calculated for $C_9H_{11}N_2O_6NaI$ ($M + Na^+$) 392.9554, found 392.9565.

6-Iodouridine 5′-Monophosphate (3). A stirred solution of H_2O (0.034 g, 1.89 mmol) and $POCl_3$ (0.28 mL, 2.97 mmol) in anhydrous acetonitrile (3 mL) was treated with pyridine (0.261 mL, 3.24 mmol) at 0 °C and stirred for 10 min. Compound **7** (0.250 g, 0.67 mmol) was added, and the mixture was stirred for an additional 5 h at 0 °C. The reaction mixture was then quenched with 25 mL of cold water and continued stirring for an additional 1 h. The evaporation of the solvent and purification of the crude by column chromatography (Dowex ion-exchange basic resin, 0.1 M formic acid) afforded compound **3** (0.207 g, 68%) as a syrup. The monophosphate compound was transformed into the ammonium salt by neutralization with 0.5 M NH_4OH solution at 0 °C and freeze-dried to obtain the ammonium salt as a pale yellow powder. UV (H_2O): λ_{max} = 267 nm (ϵ = 2890); ^{1}H NMR (D_2O) δ 3.78 (dd, 1H, H-5′), 3.91 (dd, 1H, H-5″), 3.98−4.03 (m, 1H, H-4′), 4.43 (t, 1H, H-3′), 4.84 (dd, 1H, H-2′), 6.05 (d, 1H, H-1′), 6.67 (s, 1H, H-5). ^{31}P NMR (D_2O) δ ppm 2.214. HRMS (ESI, negative) calculated for $C_9H_{11}N_2O_9PI$ (M^-) 448.9252, found 448.9263.

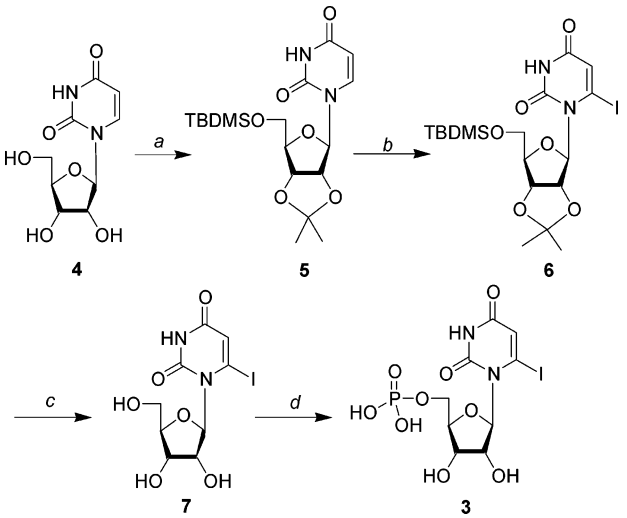
ODCase Cloning, Expression, and Purification. ODCase from *Methanobacterium thermoautotrophicum* was produced as described earlier (ref 14). The gene coding for ODCase from *Plasmodium falciparum* was cloned into a pET28 vector modified for infusion cloning and addition of an N-terminal hexahistidine affinity tag. The proteins were expressed in *E. coli* BL21 (DE3) CodonPlus-RIL in Terrific Broth (TB) in the presence of kanamycin/ chloramphenicol (50 μg/mL and 25 μg/mL, respectively). The cultures were incubated at 37 °C in a water bath with aeration and mixing provided by means of bubbling air at a rate between 4 and 6 L/min. The cultures were cooled to 15 °C when the OD_{600} reached 5. After addition of isopropyl 1-thio-D-galactopyranoside (IPTG) 0.4 mM, they were incubated at 15 °C overnight. The cultures were harvested by centrifugation, and the cell pellets were suspended in 160 mL of binding buffer (50 mM HEPES, pH 7.5, 0.5 M NaCl, 5% glycerol, and 15 mM imidazole) with protease inhibitors (1 mM benzamidine-HCl and 1 mM phenylmethylsulfonyl fluoride, PMSF) added. The suspensions were kept in 50 mL Falcon tubes at −80 °C.

Before purification, the cell suspensions were thawed overnight at 4 °C. Prior to mechanical lysis, each tube of cell suspension was pretreated with 0.5% CHAPS and 500 units of benzonase (per 40 mL of resuspended cell pellet) for 40 min at room temperature. Subsequently, the cells were mechanically lysed in a microfluidizer (Microfluidizer Processor, M-110EH) at 18 000 psi. The lysates were centrifuged at 24 000 rpm for 20 min at 10 °C. Each cleared cell lysate was loaded onto a column containing 10 g DE-52 resin (Whatman) and then directly onto a 3 mL Ni-NTA (Qiagen) column. When all the lysate was loaded, the two-column system was washed with 20 mL of binding buffer. The Ni-NTA column was then washed with 200 mL of wash buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 30 mM imidazole, and 5% glycerol). After washing, the protein was eluted from the Ni-NTA column with 15−20 mL of elution buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 250 mM imidazole, and 5% glycerol). EDTA was added immediately to 1 mM, with DTT added to 1 mM 15 min later. The mixture was dialyzed overnight in 10 mM HEPES and 500 mM NaCl. The following day, it was concentrated using a 15 mL Amicon Ultra centrifugal filter device (Millipore). Protein concentration was measured by taking absorbance at OD_{280}. Finally, aliquots of the purified protein were labeled and stored at −80 °C.

***P. falciparum* Cultures and CHO Cell Assays.** Assays comparing the antiplasmodial activities of the inhibitors were performed using the SYBR-Green method.[24] Briefly, the inhibitors were dissolved in DMSO to achieve a concentration of 10 mg/mL. Fifty microliters of RPMI-A were added to each well in a 96-well plate before 40 μL of RPMI-A and 10 μL of compound solution were added to the first well, the contents of the well were mixed, 50 μL were removed and added to the next well in the series, and the process was repeated until the next-to-last well was reached. This produced a plate with a series of 2-fold dilutions across it, except for the last well in the series, which contained RPMI-A alone. Fifty microliters of parasite culture (2% hematocrit, 2% parasitemia) were added to each well, and the plates were then incubated at 37 °C in 95% N_2, 3% CO_2, and 2% O_2 for 72 h.

CHO cells (ATCC, Manassas, VA) were grown in RPMI-1640 supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), 25 mM HEPES, and gentimicin (RPMI-10). Cells were seeded in 96-well plates and grown to 50% confluency in 100 μL of RPMI-10 per well prior to the addition of either DMSO alone, or a test compound dissolved in DMSO to a concentration of 10 mg/mL. Compound gradients were prepared by adding 90 μL of RPMI-10 mixed with 10μL of compound solution to the first well in the series, mixing, transferring 100 μL to the

Scheme 2[a]



[a] (a) Acetone/H^+; and then TBDMSCl, imidazole/CH_2Cl_2 0−25 °C; (b) LDA, I_2, THF −78 °C; (c) 50% TFA, rt; (d) $POCl_3$, pyridine, H_2O, CH_3CN, 0 °C.

next well, and repeating until the next-to-last well was reached. After 48 h, the viability of the cells was determined by discarding the media in the wells and adding 100 μL of 10 mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO) in RPMI-10, incubating the plates for an additional hour, and then removing the media and adding 100 μL of DMSO and reading the absorbance at 650 nm.[25] The IC_{50} values of individual compounds were determined by applying a nonlinear regression analysis of the dose−response curve using the computer program SigmaPlot (Jandel Scientific).

Enzymology. *Mt* ODCase and compound **3** were assayed at room temperature in the reaction buffer (50 mM Tris, 20 mM DTT, 40 mM NaCl, pH 7.5). *Pf* ODCase samples were prepared in 50 mM Tris, 10 mM DTT, 20 mM NaCl, at pH 8.0 and at room temperature. The remaining activity of ODCase was measured at 55 °C and 37 °C, for *Mt* and *Pf*, respectively, using the ITC method. Incubation mixtures contained 50 μM *Mt* ODCase or 50 μM *Pf* ODCase for time-dependent studies. In the case of *Mt* ODCase, aliquots of 1 μL were removed from the reaction mixture and diluted to 2.5 mL using the assay buffer (50 mM Tris, 1 mM DTT, pH 7.5). Each inhibition assay was initiated by an injection of 5 μL of 11.41 mM OMP (prepared in assay buffer), or coinjection of the inhibitor and OMP. The final substrate concentration was 40 μM in the assay mixture. In the case of *Pf* ODCase, a 2.5 μL aliquot of the incubation mixture was diluted with the assay buffer (50 mM Tris, 1 mM DTT, pH 8.0) to a final volume of 2.5 mL, and the reaction was initiated by a single injection of OMP or the mixture of OMP-inhibitor into the ITC sample chamber. Enzyme activity was derived from the linear portions of progress curves from the isotherms obtained from the isothermal titration calorimetry (Power vs time) using the method developed previously.[21]

Crystallographic Analysis. All *Mt* ODCase concentrations were determined using a BioRAD protein assay kit and BSA as a standard. ODCase (10 mg/mL) with 6-iodo-UMP (**3,** 10 mM) was prepared in crystallization buffer composed of 20 mM HEPES−NaOH at pH 7.5, 150 mM sodium chloride, and 5 mM DTT. This protein solution (2 μL) was mixed with 2 μL of reservoir solution containing 1.1−1.3 M sodium citrate, 5% (v/v) dioxane, and 100 mM MES at pH 6.5 and then set up for crystallization using the hanging drop technique. On the following day, microseeding started the formation of diffraction-quality crystals, which took about a week to reach their full size. When harvested, crystals were immediately dipped into mother liquor supplemented with 15% (v/v) glycerol for cryoprotection and then flash-frozen in a stream of dry nitrogen at 100 K. Diffraction data were collected to 1.6 Å resolution at beamline 14 BM-C BioCARS, Advanced Photon Source, IL. The data collected were reduced using the programs DENZO, SCALEPACK, and TRUNCATE from the CCP4 program suite.[26,27] The crystals belonged to space group $P2_1$ with unit cell parameters, a = 57.8 Å, b = 73.3 Å, c = 59.1 Å, β = 119.3°. Phases were calculated based on the coordinates of the *Mt* ODCase−BMP complex (PDB code: 1X1Z). An atomic model was constructed with the help of the program O and refined using the program CNS, version 1.1, resulting in final $R_{crystal}$ and R_{free} factors of 15.9% and 18.7%, respectively.[28,29] Data collection and final refinement statistics are shown in Table S1. Atomic coordinates and structure factors have been deposited into the Protein Data Bank under the code 2E6Y.

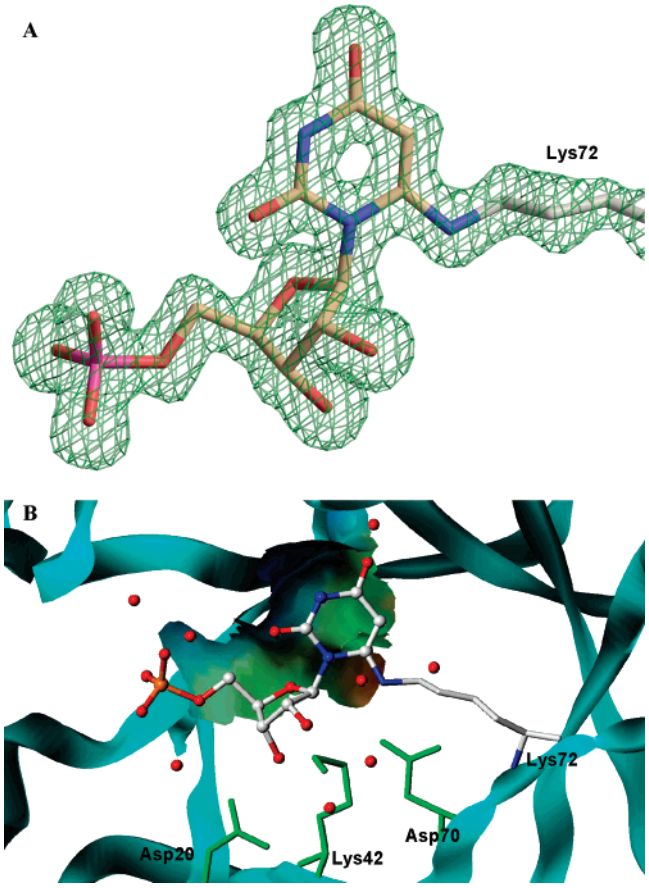


Figure 2. (A) Electron density map representing the covalent bond formed between Lys72 and C6 of compound **3** after the elimination of the iodo moiety. The $F_o - F_c$ electron density map was calculated with the corresponding atoms deleted from the model and is displayed at the 3σ level. (B) Rendering of the X-ray structure (resolution of 1.6 Å) of the covalent complex with important active site residues. The nucleotide is rendered as a ball-and-stick representation, and the key residues in the binding site of ODCase are shown in capped-stick representation in green. The backbone of ODCase is shown as a ribbon in cyan, and a portion of the binding site is rendered using a Connolly surface.

Solubility and pK_a Measurements. A supersaturated aqueous solution of compound **7** was prepared by first dissolving an excess amount of **7** in distilled, deionized water by gentle heating (~45 °C). The solution was allowed to cool to room temperature (~24 °C) and to stand for 2 days to recrystallize **7**. The

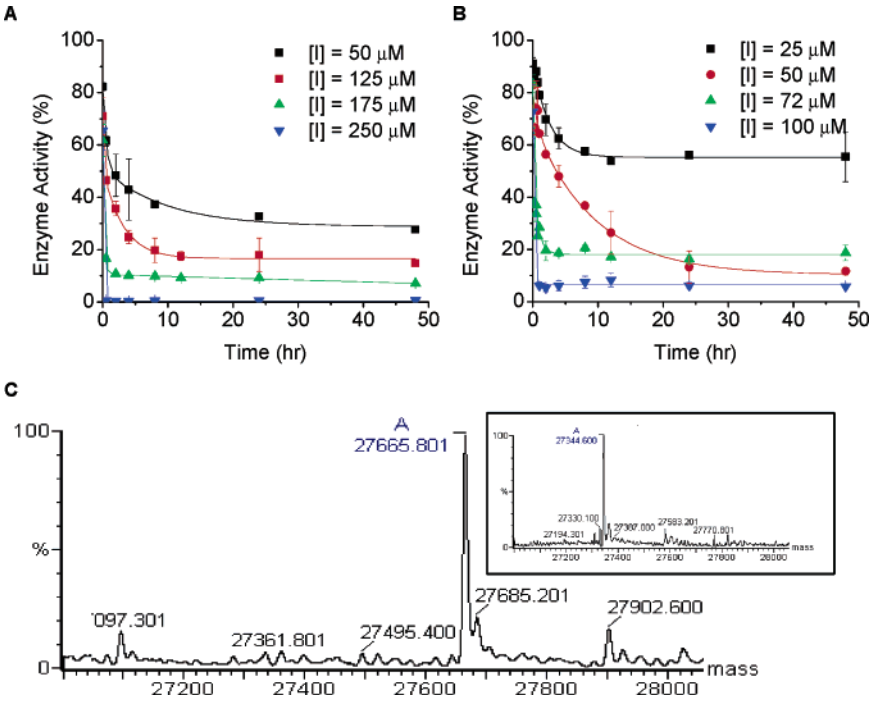


Figure 3. Profiles of the enzyme activity of *Mt* ODCase (panel A) and *Pf* ODCase (panel B) in the presence of compound **3**. (Panel C) Mass spectral analyses for the incubation mixture of ODCase and compound **3**, and ODCase alone (inset).

supernatant in equilibrium with the recrystallized compound **7** was analyzed on a Varian Cary 50 UV−vis spectrophotometer at 270 nm. The Beer's Law calibration curve for **7** was established with a series of compound dilutions in distilled, deionized water. The pK_a of **7** was determined by the method of pH titration. A 0.01 M aqueous solution of **7** was titrated with a 0.01 M NaOH standard at room-temperature using a combination pH electrode (Accumet MicroProbe) and a laboratory pH meter (Accumet AB15).

Results and Discussion

ODCase has been a target of interest for drug development due to its critical role in the *de novo* synthesis of pyrimidine nucleotides. Over the past two decades, several groups including ours have investigated various inhibitors against ODCase, the synthesis of most of which was prompted by the studies to understand the decarboxylation mechanism. As part of our investigations, we synthesized 6-iodouridine 5′-*O*-monophosphate (**3**) and investigated its interactions with ODCase.

Compound **3** was synthesized from uridine **4** (Scheme 2). Introduction of the iodo moiety at the C-6 position of protected uridine **5** was achieved using lithium diisopropylamide followed by treatment with iodine.[30] Deprotection with trifluoroacetic acid yielded 6-iodouridine (**7**), followed by phosphorylation with phosphorus oxychloride, afforded the mononucleotide **3** (Scheme 2).[31−33] Then, compound **3** was transformed into its ammonium salt by neutralization with 0.5 M NH_4OH solution at 0 °C and freeze-dried to get the ammonium salt as a powder.

When compound **3** was setup to cocrystallize with *Mt* ODCase, a covalent complex of ODCase and UMP was observed. Diffraction data were collected and analyzed to a resolution of 1.6 Å (see Supporting Information). The resulting electron density map clearly showed that the iodo-substituent had been replaced by a covalent bond between the N_ϵ of Lys72 and C-6 of the nucleotide ligand (Figure 2). As such a result was completely unexpected and quite unprecedented, we undertook extensive further analyses.

ODCase activities were investigated to understand the behavior of *Mt* ODCase in the presence of compound **3**. ODCase in general, displays much higher affinities toward mononucleotide than nucleoside analogues because the 5′-phosphate group contributes significantly to the binding energy of the ligand.[34] When *Mt* ODCase was incubated with 6-iodo-UMP (**3**), time- and concentration-dependent loss of enzyme activity was observed (Figure 3A). *Mt* ODCase lost more than 60% of its catalytic activity at [I] = [E] (50 μM) in the first 8 h, and the activity was not recovered even after 96 h. Experiments with increased concentrations of **3** revealed that *Mt* ODCase was completely inhibited at 250 μM (5 × [E]) concentration of the inhibitor in less than 30 min (Figure 3A). The rate of inactivation was very fast even at low concentrations of the enzyme and inhibitor **3** ([I] = [E] = 50 μM), and the initial rates could not be obtained reliably with the existing enzyme assay method using ITC for additional kinetics characterization.[21]

To further confirm the data, mass spectral analyses were undertaken with the *Mt* ODCase−inhibitor complex. Compound **3** was incubated with *Mt* ODCase overnight at room temperature. A high-resolution mass spectral analysis showed that the iodo moiety was eliminated, and the total mass of the molecular species was equivalent to that of the complex of *Mt* ODCase with inhibitor, but without the iodo moiety (MW 27 665.8 Daltons) (Figure 3C). A control experiment with *Mt* ODCase without inhibitor **3** showed only the intact enzyme (MW 27, 344.6 Da) (inset in Figure 3C). A closer examination of the mass spectra revealed that the total mass of the enzyme−inhibitor complex corresponded to a loss of two extra protons, in addition to the loss of the iodo group. These two mass units could be attributed to the loss of two protons of the (protonated) amine of the Lys residue in the catalytic site. This Lys residue was covalently linked to the C-6 position of the inhibitor **3**, thus satisfying the valency at the C-6 carbon. This is in excellent agreement with the three-dimensional structure determined using X-ray crystallography (*vide supra*).

Table 1. *In Vitro* Cell-Based Activities of Compound **7**

	IC_{50} (μM)
P. falciparum ItG	4.4 ± 1.3
P. falciparum 3D7	6.2 ± 0.7
CHO cells	366 ± 45

A control experiment on the stability of compound **3** without *Mt* ODCase was conducted to rule out any chemical instability. Compound **3** was incubated in water at 55 °C for 1 h, and the reaction was analyzed using thin layer chromatography and mass spectrometry. There were no traces of either hydrolyzed product (BMP) or other decomposition products. The purity of the inhibitor **3** was further confirmed by HPLC, and it is more than 99.3% pure (see Supporting Information), eliminating the possibility of the inhibitor sample containing a reactive contaminant.

This is the first example of a covalent inhibitor for ODCase, an enzyme which does not use covalent interactions with the substrate during its physiologically relevant catalysis. This finding is especially interesting because (i) none of the residues in the binding site of ODCase are known to be nucleophilic, (ii) ODCase catalysis does not involve any covalent species, and (iii) the overall active site architecture of ODCase, especially the tight hydrogen-bonding network, precludes any such nucleophilic environment. In fact, the active site has evolved to stabilize C-6 "electron-rich" ligands and/or transition-state species.[1,20]

Following these findings, we were interested in evaluating compound **3** as a potential inhibitor of the therapeutically important homologue *Pf* ODCase. This is also motivated by the comparison of the three-dimensional structures of *Mt* ODCase and *Pf* ODCase, which are identical in the substrate binding site (ref 14; and PDB IDs 2FDS, 2F84, 2AQW). *Pf* ODCase activity was monitored in the presence of compound **3** (Figure 3B). The pattern of inactivation is very similar to that observed with *Mt* ODCase, except *Pf* ODCase appeared to be more sensitive to compound **3**. Thus, both enzymes showed similar inactivation behavior when challenged with inhibitor **3** at nanomolar to micromolar concentrations range.

Due to the potency of this compound against *Pf* ODCase, it was of further interest to evaluate the *in vitro* potential of 6-iodouridine (**7**) (parent nucleoside analogue of **3**) for antiplasmodial activities. Compound **3** is a monophosphate derivative with little potential of exhibiting any cell-based activities or being useful as a potential drug candidate. Thus, we evaluated its nucleoside analogue **7** against chloroquine-resistant and chloroquine-sensitive *P. falciparum* isolates *in vitro* (ItG and 3D7, respectively). Compound **7** exhibited potent activity against these two strains with an IC_{50} of 4.4 ± 1.3 and 6.2 ± 0.7 μM, respectively (Table 1). Compound **7** exhibited an IC_{50} of 366 ± 45 μM against CHO cell lines. There is approximately 2 orders of magnitude difference between the *in vitro* antiplasmodial activities and cell toxicity based on the above results. Equilibrium solubility evaluation indicated that compound **7** has respectable solubility in distilled water at 4.32 mg/mL. A subsequent pK_a study indicated that compound **7** has a pK_a of 8.42 based on the inflection point in the pH titration curve. This preliminary physicochemical characterization of **7** along with encouraging antiplasmodial activities place compound **7** among the interesting compounds to be considered for further development as an antimalarial agent.

Here, we reveal 6-iodo-UMP (**3**) as the first covalent inhibitor of ODCase. The covalent bond between the ligand and the enzyme is via catalytic residue Lys72 (*Mt* ODCase numbering). This inhibitor also inhibits *P. falciparum* ODCase irreversibly, and its nucleoside analogue, 6-iodouridine (**7**), exhibits potent *in vitro* antiparasitic activities against *P. falciparum* including a chloroquine-resistant isolate. The findings disclosed here are unique and should rejuvenate the interest of medicinal chemists to consider ODCase as a potential antiparasitic drug target using novel nucleoside inhibitors.

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Supporting Information Available: Details of data collection, structure determination, and refinement of the *Mt* ODCase−UMP covalent complex; HPLC purity data on compounds **7** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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