Electronic Supplementary Information (ESI) for

Interrogation of the Active Site of OMP Decarboxylase from Escherichia coli with a Substrate Analogue Bearing an Anionic Group at C6

Srinath Thirumalairajan, a Brandi Mahaney, a and Stephen L. Bearne a,b,*

aDepartment of Biochemistry & Molecular Biology, Dalhousie University, Halifax, Nova Scotia, B3H 1X5

bDepartment of Chemistry, Dalhousie University, Halifax, Nova Scotia, B3H 1J3

*To whom correspondence should be addressed (sbearne@dal.ca)

Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>General experimental information</td>
<td>S2</td>
</tr>
<tr>
<td>Synthesis of compounds 5–10</td>
<td>S2–S9</td>
</tr>
<tr>
<td>Sub-cloning of the open reading frame encoding E. coli ODCase</td>
<td>S9–S11</td>
</tr>
<tr>
<td>Overproduction and purification of the EcODCase</td>
<td>S11–S12</td>
</tr>
<tr>
<td>Enzyme assays</td>
<td>S12–S14</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>S14–S16</td>
</tr>
<tr>
<td>Table S1: Reported $K_i$ and $pK_a$ values for inhibitors mentioned in the text</td>
<td>S16</td>
</tr>
<tr>
<td>References</td>
<td>S16</td>
</tr>
<tr>
<td>NMR spectra</td>
<td>S18–S40</td>
</tr>
</tbody>
</table>

NMR spectra

Supplementary Information
**General.** All starting materials and reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Tetrahydrofuran (THF) was distilled from sodium metal in the presence of benzophenone under argon. Dichloromethane (DCM) was distilled from calcium hydride under argon. Petrol refers to petroleum ether (b.p. 40 – 60 °C) and ether refers to diethyl ether unless stated otherwise. All solvents were purchased from Fisher Scientific (Ottawa, ON, Canada). Trimethyl phosphite was freshly distilled before use. Acetonitrile was distilled from phosphorus pentoxide and then from potassium carbonate and stored over molecular sieves (4 Å). Flash column chromatography was conducted using silica gel (60 Å, 70-230 mesh) purchased from Rose Scientific Ltd. (Edmonton, AB, Canada). $^1$H-, $^{13}$C-, and $^{31}$P-NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer. For $^1$H-NMR in CDCl$_3$, chemical shifts (δ) are reported in ppm relative to the solvent residual peak (δ 7.26). For $^1$H-NMR in MeOD, chemical shifts are reported in ppm relative to tetramethylsilane (δ 0.0, internal standard). For $^1$H-NMR in D$_2$O, chemical shifts are reported in ppm relative to the solvent residual peak (δ 4.79). For $^{13}$C-NMR in CDCl$_3$, the chemical shifts are reported in ppm relative to CDCl$_3$ (δ 77.36 for the central peak). All $^{31}$P-NMR chemical shifts are reported relative to 85% H$_3$PO$_4$ in D$_2$O (δ 0.0, external standard). NMR data are reported as follows: app., apparent; br, broad; s, singlet; d, doublet; t, triplet; q, quartet; dd, double doublet, qt, quintet; and m, multiplet. Low resolution (LR) and high resolution (HR) electrospray ionization (ESI) mass spectra (MS) were obtained using a Bruker microTOF mass spectrometer operating in either the positive or negative ion mode as indicated in the experimental procedures below. High resolution (HR) electron impact ionization (EI) mass spectra were obtained using a CEC 21-110B mass spectrometer operated at a mass resolution of 8000 (10% valley) by computer controlled peak matching to appropriate perfluoro kerosene (PFK) ions. Elemental analyses of the compounds were performed by Canadian Microanalytical Service Ltd. (Delta, BC, Canada).

**2′,3′-O-Isopropylideneuridine.** To a solution of uridine (4.88 g, 20 mmol) and a catalytic amount of p-toluenesulfonic acid (TsOH·H$_2$O, 0.380 g, 2.0 mmol) in acetone (120 mL), 2,2-
dimethoxypropane (2.65 mL, 2.24 g, 21.8 mmol) was added at 0 °C. The suspension was slowly heated to a gentle reflux which was maintained for 1 h. The solution was then cooled to room temperature and treated with sodium bicarbonate (0.3036 g, 3.6 mmol) and stirred at room temperature for an additional 0.5 h. The solid was filtered and the filtrate was adsorbed on silica gel and purified by flash chromatography (gradient of 5–10% methanol in DCM) to give 5.69 g (100%) of the title compound as a white crystalline solid. \( R_f = 0.27 \) (10% MeOH in DCM); mp 165-167 °C (lit. 163-164 °C); \(^1\)H-NMR (MeOD, 500 MHz) \( \delta \) (ppm): 7.82 (1H, d, \( J_{6,5} = 8.05 \) Hz, 6-CH), 5.86 (1H, d, \( J_{1',2'} = 2.7 \) Hz, 1'-CH), 5.67 (1H, d, \( J_{5,6} = 8.04 \) Hz, 5-CH), 4.19 (1H, app. t, \( J_{2',3'} = 3.70 \) Hz, 2'-CH), 3.54 (3H, m, 5'-CH\(_2\) & 4'-CH), 3.30 (1H, m, 3'-CH), 1.54 & 1.34 (3H each, s, 2 \( \times \) CH\(_3\)); \(^13\)C-NMR (MeOD, 500 MHz) \( \delta \) (ppm): 164.8 (4-CO), 150.7 (2-CO), 142.4 (6-CH), 113.7 (C(CH\(_3\))\(_2\)), 101.3 (5-CH), 92.7 (1'-CH), 87.0 (2'-CH), 84.4 (4'-CH), 80.8 (3'-CH), 61.7 (5'-CH\(_2\)), 26.1 (CH\(_3\)) & 24.1 (CH\(_3\)); LRMS (ESI\(^+\), MeOH) \( m/z \): 307.1 [(M+Na)\(^+\), 100%], 591.2 [(2M+Na)\(^+\), 60%], 875.3 [(3M+Na)\(^+\), 30%]; HRMS (ESI\(^+\), MeOH) \( m/z \): Found 307.0898; calculated 307.0901 for C\(_{12}\)H\(_{16}\)N\(_2\)NaO\(_6\) [M+Na]\(^+\).

5'-O-tet-Butyldimethylsilyl-2',3'-O-isopropylideneuridine (6a). The title compound was prepared according to the method described by Fujihashi et al.\(^2\) A solution of 2',3'-O-isopropylideneuridine (5.12 g, 18.0 mmol) in anhydrous DCM was treated with imidazole (2.45 g, 36.0 mmol) and TBSCl (3.12 mL, 2.72 g, 18.0 mmol) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 1.5 h. When the reaction was complete (TLC), it was diluted with DCM and the organic layer was washed with water and brine and then dried over MgSO\(_4\). The solvent was evaporated in vacuo and the crude product was purified by flash chromatography (gradient of 30–50% EtOAc in petrol) to give 7.0 g (99%) of the TBS ether 6a as a white solid. \( R_f = 0.33 \) (40% EtOAc in hexanes); \(^1\)H-NMR (CDCl\(_3\), 500 MHz) \( \delta \) (ppm): 9.08 (1H, br s, NH), 7.71 (1H, d, \( J_{6,5} = 8.12 \) Hz, 6-CH), 6.01 (1H, d, \( J_{1',2'} = 2.76 \) Hz, 1'-CH), 5.71 (1H, dd, \( J_{5,6} = 8.1 \) Hz, 5-CH), 4.79 (1H, dd, \( J_{2',3'} = 6.15 \) Hz, 2'-CH), 4.72 (1H, dd, \( J_{3,2'} = 6.15 \) Hz, 3'-CH), 4.34 (1H, app. d, \( J_{3',3',5'} = 2.65 \) Hz, 4'-CH), 3.95 (1H, app. q, \( J_{5,a,5b,d'} = 2.41 \) &
11.5 Hz, 5′-CH$_{2(o)}$, 3.82 (1H, app.q, J$_{5b,5a,4′}$ = 3.0 & 11.5 Hz, 5′-CH$_{2(0)}$), 1.61 & 1.38 (3H each, s, 2 × -C(CH$_3$)$_3$), 0.93 (9H, s, SiBu-t), 0.1 (6H, s, Si-CH$_3$); $^{13}$C-NMR (CDCl$_3$, 500 MHz) δ (ppm): 163.3 (4-CO), 150.1 (2-CO), 140.6 (6-CH), 114.2 (-C(CH$_3$)$_2$), 102.2 (5-CH), 91.9 (1′-CH), 86.7 (2′-CH), 85.4 (4′-CH), 80.3 (3′-CH), 63.4 (5′-CH$_2$), 27.3 & 25.9 (-C(CH$_3$)$_2$), 25.4 (-C(CH$_3$)$_3$), 18.4 (-C(CH$_3$)$_3$), -5.4 and -5.1 (-Si-CH$_3$); LRMS (ESI⁺, MeOH) m/z: 818.8 [(2M+Na)$^+$, 100%], 421.1 [(M+Na)$^+$, 35%].

$^{5′-O}$-O-tert-Butyldimethylsilyl-$2′,3′$-O-isopropylidine-$6$-diethylphosphonouridine (7a). The title compound was prepared by following the literature procedure reported by Honjo et al.$^3$ with some minor modifications. To a solution of diisopropylamine (0.35 mL, 0.252 g, 2.5 mmol) in THF, 1.45 mL of $n$-butyllithium (1.8 M solution in hexanes) was added in a dropwise manner at −78 °C. The mixture was maintained with stirring at −78 °C for 1 h under argon. To this, a solution of the TBS protected compound 6a (0.398 g, 1.0 mmol) in THF was then added dropwise at −78 °C over a period of 5-10 min and the resultant mixture was maintained at −78 °C for 1 h. Then, diethyl chlorophosphate (0.43 mL, 0.52 g, 3.0 mmol) was added dropwise at −78 °C over a period of 10 min and the reaction was maintained at this temperature for 2 h. When the reaction was complete (TLC), aqueous saturated ammonium chloride was added slowly to quench the reaction. The aqueous layer was then extracted with EtOAc. The combined organic layer was then washed well with water and then dried over MgSO$_4$. The solvent was evaporated in vacuo and the crude product was adsorbed on silica gel and purified by flash chromatography (gradient of 15–50% EtOAc in petrol) to give 0.180 g (33%) of compound 7a as colourless viscous liquid. $^1$H-NMR (CDCl$_3$, 500 MHz) δ (ppm): 9.82 (1H, br s, -NH), 6.44 (1H, d, J$_{1′,2′}$ = 4.03 Hz, 1′-CH), 6.14 (1H, s, 5-C-CH), 5.16 (1H, app. d, J$_{2′,3′}$ = 6.5 Hz, 2′-CH), 4.76 (1H, app. t, J$_{3′,4′}$ = 4.92 & 6.02 Hz, 3′-CH), 4.25 (4H, m, 2 × -OCH$_2$CH$_3$), 4.09 (1H, app. q, J$_{4′,3′,5′}$ = 5.2 & 11.6 Hz, 4′-CH), 3.79 (2H, m, 5′-CH$_2$), 1.51 & 1.31 (3H each, s, -C(CH$_3$)$_2$), 1.39 (6H, t, J$_{H-H}$ = 7.04 Hz, 2 × -OCH$_2$CH$_3$), 0.85 (9H, s, SiBu-t), 0.01 (6H, s, Si-CH$_3$); $^{13}$C-NMR (CDCl$_3$, 500 MHz) δ (ppm): 162.3 (d, 4-CO), 150.4 (d, 2-CO), 146.1 (d, 6-CPO), 114.0 (s, -C(CH$_3$)$_2$), 112.4
Supplementary Information

5′-O-(Tetrahydro-2-furanyl)-2′,3′-O-isopropylideneuridine (6b). The title compound was prepared following the method reported by Honjo et al.\textsuperscript{3} 2,3-Dihydrofuran (2.9 mL, 2.7 g, 38.35 mmol) and PPTS (0.503 g, 2 mmol) were added to a solution of 2′,3′-O-isopropylideneuridine (5.68 g, 20 mmol) in anhydrous DCM. After standing at room temperature for 1 h, the reaction went to completion (TLC). The mixture was then concentrated in vacuo and purified by flash chromatography (gradient of 2–5% methanol in DCM) to give 7.0 g (100%) of a viscous oil which formed a colorless, hygroscopic solid upon standing. \( R_f = 0.4 \) (10% MeOH in DCM); \( ^1\text{H}-\text{NMR} (\text{CDCl}_3, 500 \text{ MHz}) \) \( \delta \) (ppm): 9.54 (1H, br s, NH), 7.57 (1H, d, \( J_{6,5} = 7.6 \text{ Hz}, 6-\text{CH} \)), 7.55 (1H, d, \( J_{6,5} = 7.6 \text{ Hz}, 6-\text{CH} \)), 7.85 (1H, d, \( J_{1,2'} = 2.20 \text{ Hz}, 1'-\text{CH} \)), 5.67 (1H, d, \( J_{5,6} = 8.04 \text{ Hz}, 5-\text{CH} \)), 5.10 (1H, app. q, 2′-CH), 4.75-4.71 (2H, m, 3′-CH, 1′′-CH), 4.41-4.34 (1H, m, 4′-CH), 3.97-3.51 (5H, m, 5′-CH\( _2 \) & 4″-CH\( _2 \)), 1.97-1.84 (6H, m, 2′′, 3′′ & 4″-CH\( _2 \)), 1.56 & 1.33 (3H each, s, 2 × CH\( _3 \)); \( ^{13}\text{C}-\text{NMR} (\text{CDCl}_3, 500 \text{ MHz}) \) \( \delta \) (ppm): 163.6 (4-CO), 150.2 (d, 2-CO), 141.0 (d, 6-CH), 114.1 (d, C(CH\( _2 \))\( _2 \)), 104.0 (d, 1′′-CH), 102.0 (d, 5-CH), 93.0 (d, 1′-CH), 85.3 (d, 2′-CH), 85.0 (d, 3′-CH), 81.0 (d, 4′-CH), 67.3 (d, 4″-CH\( _2 \)), 67.0 (d, 5′-CH\( _2 \)), 32.4 (d, 2″-CH\( _2 \)), 27.2 & 25.3 (2 × d, 2 × CH\( _3 \)) and 23.4 (d, 3″-CH\( _2 \)); LRMS (ESI\(^+\), MeOH) \( m/z \): 754.1 [2(M+Na)\(^+\), 35%], 731.0 [(2M+Na)\(^+\), 55%], 377.2 [(M+Na)\(^+\), 100%].

Diethyl 2′,3′-O-isopropylidinuridine-6-phosphonate, 8: Step 1, synthesis of 7b. This compound was prepared according to the procedure reported by Honjo et al.\textsuperscript{3} with some minor modifications. To a solution of diisopropylamine (3.7 mL, 2.67 g, 26.0 mmol) in THF, cooled to −78 °C and under argon, \( n \)-butyllithium (14.5 mL of 1.8 M solution in hexane, 26.0 mmol) was
added dropwise at –78 °C. The mixture was then stirred at –78 °C for 1 h. A solution of compound 8 (3.55 g, 10.0 mmol) in THF was then added dropwise to the mixture at –78 °C. The mixture was stirred for 1 h and then diethyl chlorophosphate (4.75 ml, 5.7 g, 33 mmol) was added dropwise at –78 °C when the colour of the solution changed from yellow to dark brown. The solution was maintained at this temperature for 1 h and after completion of the reaction (TLC), the reaction was quenched by the addition of aqueous saturated solution of ammonium chloride. The aqueous layer was then extracted with ethyl acetate, the combined organic layer was then washed with water and the solvent evaporated in vacuo to give ~5.0 g of yellowish-brown viscous liquid which was taken for the deprotection step without any further purification.

**Step 2, synthesis of 8.** The product from the above step was dissolved in EtOH (20 mL), and PPTS (0.26 g, 1 mmol) was added. After standing at room temperature for 0.5 h to 0.75 h (TLC), the solvent in the reaction mixture was evaporated in vacuo. The resultant crude product was adsorbed on silica gel and purified by flash chromatography (gradient of 60–100% ethyl acetate in petrol) to give 3.93 g of 8 as a pale yellow viscous oil. Yield – 70%; R_f = 0.32 (10% MeOH in DCM); ¹H-NMR (CDCl₃, 500 MHz) δ (ppm): 10.50 (1H, br s, NH), 6.44 (1H, d, J_{HCCP} = 13.1 Hz, 5′-CH), 6.10 (1H, d, J_{5,5'} = 2.1 Hz, 1′-CH), 5.23 (1H, q, J_{2,3'} = 6.6 Hz, 2′-CH), 5.01 (1H, q, J_{3,4'} = 6.5 Hz, 3′-CH), 4.25 (5H, m, 5′-OH & 2 × CH₂CH₃), 4.10 (1H, m, 4′-CH), 3.85 (1H, q, J_{5a,4} = 3.0 Hz & J_{5a,5b} = 12.07 Hz, 5′a-CH₂), 3.75 (1H, q, J_{5b,4} = 3.9 Hz, 5′b-CH₂), 1.53 & 1.32 (3H each, s, 2 × CH₃), 1.38 (6H, sestet, 2 × CH₂CH₃); ¹³C-NMR (CDCl₃, 500 MHz) δ (ppm): 171.23 (4′-CO), 161.8 (2′-CO), 151.2 (6′-C), 114.2 (C(CH₃)₂), 112.6 (5′-CH), 94.0 (1′-CH), 87.2 (2′-CH), 83.7 (4′-CH), 80.4 (3′-CH), 64.5 and 62.4 (2 × CH₂CH₃), 60.4 (5′-CH₂), 27.1 and 25.2 (2 × CH₃), 16.1 & 14.1 (2 × CH₂CH₃); ³¹P-NMR (CDCl₃, 500 MHz) δ (ppm): 5.60 (app. septet); LRMS (ESI⁺, MeOH) m/z: 907.1 [(2M+3Na)⁺, 52%], 886 [2(M+Na)⁺, 30%], 862.8 [(2M+Na)⁺, 23%], 443.1 [(M+Na)⁺, 100%]; HRMS (ESI⁺, MeOH ) m/z: Found 443.1192; calculated 443.1190 for C_{16}H_{25}N₂NaO₉P [M+Na]⁺.

**Diethyl 2′,3′-O-isopropylidene-6-diethylphosphonouridin-5′-dimethyl phosphate, 9.** The title compound was prepared using a method similar to that described by Oza et al.⁴ with minor
modifications. Carbon tetrabromide (CBr₄) (2.92 g, 8.8 mmol) and compound 8 (1.65 g, 4.0 mmol) were added to an argon-purged flask equipped with a magnetic stirring bar. After addition of anhydrous pyridine (12 mL) to the reaction mixture, the resulting solution was cooled to 0 °C. Freshly distilled trimethyl phosphite (TMP) (1.2 mL, 12 mmol) was then added dropwise to the mixture. (Note: A yellow-orange precipitate was formed). The mixture was maintained at 0 °C and the reaction was complete within 0.5 h (TLC). After dilution with chloroform, pyridine was evaporated in vacuo from the reaction mixture. The process was repeated three more times by adding EtOH (15-20 mL) to the reaction mixture. The crude product was then adsorbed onto silica gel and purified by flash chromatography (gradient of 80–100% EtOAc in petrol) to give 1.6 g of compound 9 as colourless viscous oil. Yield – 76%; Rₛ = 0.35 (10% MeOH in DCM) ; ¹H-NMR (CDCl₃, 500 MHz) δ (ppm): 10.21 (1H, br s, NH), 6.39 (1H, d, J₅,₆ = 2.3 Hz, 1'-CH), 5.15 (1H, q, J₂,₃ = 6.5 Hz, 2'-CH), 4.85 (1H, q, J₃,₄ = 6.5 Hz, 3'-CH), 4.23 (7H, m, 5'-CH₂, 4'-CH & 2 × -OCH₂CH₃), 3.71 (6H, dd, J₅OPOCH = 11.2 Hz, 2 × -OCH₃), 1.49 & 1.29 (3H each, s, 2 × -CH₃), 1.37 (6H, t, J = 7.0 Hz, 2 × -CH₂CH₃); ¹³C-NMR (CDCl₃, 500 MHz) δ (ppm): 162.0 (d, 4'-CO), 150.4 (d, 2'-CO), 145.2 (d, 6'-C), 113.9 (d, -C(CH₃)₂), 112.2 (d, 5'-CH), 94.7 (d, 1'-CH), 87.2 (d, 2'-CH), 84.6 (3'-CH), 82.1 (4'-CH), 67.5 (d, 5'-CH₂), 64.7 & 64.5 (dd, 2 × -OCH₂CH₃), 54.4 (app. t, 2 × -OCH₃), 26.9 & 25.2 (2 × -CH₃), 16.0 (app. t, 2 × -OCH₂CH₃); ³¹P-NMR (CDCl₃, 500 MHz) δ (ppm): 5.62 (app. septet, -PO) and 1.02 (m, 6-CPO); LRMS (ESI⁺, MeOH ) m/z: 1078.7 [(2M+Na)⁺, 20%], 551.1 [(M+Na)⁺, 100%]; HRMS (ESI⁺, MeOH ) m/z: Found 551.1158; calculated 551.1166 for C₁₈H₃₀N₂NaO₁₂P₂[M+Na]⁺.

6-phosphono uridine-5'-phosphate (10). To a solution of compound 9 (0.264 g, 0.5 mmol) in anhydrous acetonitrile under argon, bromotrimethylsilane (1.15 g, 0.97 mL, 7.5 mmol) was added in a dropwise manner using a syringe at 0 °C. After completion of the addition, the mixture was allowed to warm to room temperature and was maintained at room temperature for 24 h (TLC; phosphonate/phosphate detected using a sulfosalicylic acid-iron (III) chloride spray
reagent). The solvent was then evaporated in vacuo followed by the addition of methanol which was subsequently evaporated. This process was repeated at least three more times to give a pale yellow solid which was purified by anion exchange chromatography (DEAE Sephadex A-25, Cl⁻-form) using a linear gradient of triethylammonium bicarbonate (TEAB, 0.1 – 0.5 M) buffer (pH 7.5 – 8.0) to give three UV (265 nm) active fractions that were lyophilized to constant weight to give the title compound (last fraction) along with monoethyl phosphonate (11) (second fraction) and other unidentified minor impurities (first fraction) as their triethylammonium salts. Compound 10 was converted to its sodium salt by passing it through a cation exchange resin (BioRad AG-1X8, Na⁺-form) to yield the final product (0.051 g, 25%).

Compound 10: ¹H-NMR (D₂O, 500 MHz) δ (ppm): 6.53 (1H, app. d, J₁/₂\,=\,2.2\,Hz, 1'-CH), 6.26 (1H, d, J_{HCCP} = 10.66 Hz, 5-CH), 4.69 (1H, app. q, J₂/₃\,=\,6.53\,Hz, 2'-CH), 4.29 (1H, app. t, J₃/₄\,=\,7.3 & 7.0 Hz, 3'-CH), 4.12-3.97 (3H, m, 5'-CH₂ & 4'-CH); ¹³C-NMR (D₂O, 500 MHz) δ (ppm): 166.7 (d, 2-CO), 159.3 (d, 6-C), 106.3 (d, 5-CH), 94.7 (d, 1'-CH), 81.8 (d, 4'-CH), 72.0 (s, 2'-CH), 69.6 (s, 3'-CH), 64.9 (d, 5'-CH₂); ³¹P-NMR (D₂O, 500 MHz) δ (ppm): 0.93 (app. t, J₅/₆\,=\,5.8 & 5.69 Hz, 5'-OPO) and −0.85 (app. d, J₆/₇\,=\,10.54 Hz, 6-CPO); LRMS (ESI⁻, H₂O ) m/z: 402.9 (M⁻, 80%), 190.9 (M-base), 100%); HRMS (ESI⁻, H₂O) m/z: Found 402.9949; calculated 402.9949 for C₉H₁₃N₂O₁₂P₂ [M⁻]; Elemental Analysis (%): Found C, 20.22; H, 3.31; N, 5.12; and P, 11.84. Calculated C, 20.24; H, 3.06; N, 5.24; P, 11.60; Na, 14.63; and O, 45.23 for C₅H₁₆₂Na₃O₁₅P₂ (containing 3.4 Na and 3.1 H₂O).

Compound 11: Yield – 0.010 g, 5%; ¹H-NMR (D₂O, 500 MHz) δ (ppm): 6.37 (1H, d, J_{HCCP} = 12.0 Hz, 5-CH), 6.26 (1H, d, J₁/₂\,=\,2.3\,Hz, 1'-CH), 2'-CH was hidden under the solvent peak but was identified using 2D-COSY and HMQC spectra, 4.52 (1H, app. t, J₃/₄\,=\,7.3\,Hz, 3'-CH), 4.15 (1H, m, 4'-CH), 4.07 (4H, m, 5'-CH₂ & -OCH₂CH₃), 1.35 (3H, t, J = 7.1 Hz, -OCH₂CH₃); ¹¹C-NMR (D₂O, 500 MHz) δ (ppm): 165.2 (d, 4-CO), 151.6 (app. t, 2-CO & 6-C), 109.3 (d, 5-CH), 95.0 (d, 1'-CH), 81.8 (d, 4'-CH), 70.63 (s, 2'-CH), 69.3 (s, 3'-CH), 64.4 (d, 5'-CH₂), 62.9 (d, -OCH₂CH₃), 15.7 (d, -OCH₂CH₃); ³¹P-NMR (D₂O, 500 MHz) δ (ppm): 1.83 (app. t, J₅/₆\,=\,5.5 & 4.9 Hz, 5'-OPO) and 0.68 (m, 6-CPO); LRMS (ESI⁻, H₂O ) m/z: 884.9 [(2M+Na⁻), 23%], 862.2
[2M⁺, 57%] 431.1 [M⁺, 100%]; HRMS (ESI, H₂O) m/z: Found 431.0259; calculated 431.0262 for C₁₁H₁₇N₂O₁₁P₂ [M⁺].

Sub-cloning of the open reading frame encoding E. coli ODCase (EcODCase). E. coli DH5α cells were used for cloning and BL21 (DE3) cells were used for protein expression. Plasmids were isolated from 5-mL overnight cultures using a QIAprep Miniprep Kit (Qiagen, Mississauga, ON, Canada) and following the directions of the manufacturer. All cultures were grown in LB (Luria-Bertani) broth supplemented with ampicillin (100 µg/mL) or on LB agar plates containing ampicillin (100 µg/mL) unless otherwise noted.

The open reading frame (ORF) from the pyrF gene (SWISS-PROT accession number P08244) coding for E. coli ODCase was PCR-amplified from E. coli chromosomal DNA using the following synthetic deoxyoligonucleotide primers: 5′-GGTCTGCATATGACGTTAACTGCTT-CATC-3′ (forward) and 5′-GTTGGAATGGATCCTCATGCACCTCCGCTG-3′ (reverse). The forward and reverse primers included NdeI and BamHI recognition sites (underlined), respectively, for insertion of the doubly digested PCR product into the multiple cloning site of the pET15b expression vector (Novagen Inc., Madison, WI, USA), generating the pET15b-EcODCase plasmid. This plasmid encodes ODCase as a fusion protein bearing an N-terminal hexahistidine tag (His₆-tag) with an intervening thrombin cleavage site.

Template DNA was obtained from E. coli genomic DNA isolated from the DH5α strain. An overnight culture (1.5 mL) of E. coli DH5α cells was re-suspended in Tris-EDTA (TE) buffer (600 mL; 10 mM Tris-Cl, 0.1 mM EDTA, pH 8.0) containing proteinase K (100 µL/mL).
and SDS (0.5%) and incubated for 1 h at 37 °C. The salt concentration was increased to 0.7 M with 2 M NaCl. Protein was precipitated by adding a solution of hexadecyltrimethylammonium bromide (CTAB) and sodium chloride (102 mL; 10% CTAB, 0.7 M NaCl) and incubating for 10 min at 65 °C. The solution was extracted twice with chloroform/isoamyl alcohol (24:1) and the DNA in the aqueous layer was precipitated by adding 0.6 volumes of isopropanol. The DNA pellet was washed with 70% ethanol and re-suspended in TE (50 mL; 10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

The ORF was amplified using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA) and the following polymerase chain reaction (PCR) protocol: 1 min at 95 °C; 29 cycles of 1 min at 95 °C, 2 min at 50 °C, and 4 min at 72 °C; and a final extension of 4 min at 72 °C. The PCR product was purified using a QIAquick PCR Purification Kit (Qiagen). The pET-15b plasmid and the PCR-amplified ORF were each doubly digested with the restriction enzymes NdeI and BamHI (New England Biolabs, Ipswich, MA, USA). BamHI was added after digestion with NdeI and the concentration of NaCl in the digestion was increased to 150 mM with 2 M, sterile NaCl. The pET15b plasmid was also treated with alkaline phosphatase (New England Biolabs) for 1 h after the double digestion. The digested plasmid was then purified using a QIAquick PCR Purification Kit (Qiagen). The ORF was ligated into the doubly digested, phosphatase-treated pET15b plasmid overnight at 15 °C using T4 DNA ligase (Invitrogen, Burlington, ON, Canada) in a ratio of 90 fmol insert to 30 fmol vector. The ligation reaction (20 µL) was stopped by adding 0.5 M EDTA (1 µL) and then diluted 5-fold. This diluted ligation mixture (3 µL) was used to transform 100 µL of chemically competent E. coli DH5α cells. The transformation mixture was left on ice for 45 min, heat shocked for 3 min at 42 °C, and placed back on ice for 5 min before plating. Colonies were selected and examined for the ODCase ORF insert by digesting the recovered plasmids with NdeI, and, when necessary, by double digesting with BamHI and NdeI. The cut plasmids were analyzed using agarose gel (1%) electrophoresis. Insertion of the correct sequence was verified by commercial DNA sequencing (Center for Functional Microbial Genomics and Host Defence, Halifax, NS, Canada). Competent E. coli
BL21 (DE3) cells were transformed with the pET15b-EcODCase vector as previously described for the DH5α strain.

**Production and purification of EcODCase.** Starter cultures of the *E. coli* BL21 (DE3) cells containing the pET15b-EcODCase vector were grown overnight at 37 °C in LB broth (2 mL) containing ampicillin (100 μg/mL) and used to inoculate LB broth (1.0 L) containing ampicillin. Protein expression was induced by the addition of isopropyl-β-thiogalactopyranoside (IPTG, 0.1 mM) to the culture when the OD₆₀₀ reached a value between 0.5-0.7. The cultures were grown for an additional 5-7 h and then the cells were harvested by centrifugation (4000 × g, 15 min, 4 °C) and washed with binding buffer (5 mM imidazole, 250 mM NaCl, 20 mM Tris-Cl, 10% v/v glycerol, 5 mM UMP, pH 7.9). The cell pellets were frozen with liquid nitrogen and stored at –20 °C. To purify the enzyme, the cell pellets were re-suspended in binding buffer and lysed using sonication (Branson Sonifier 250; 5 × 10 s burst, duty cycle 5, constant output). The cell lysate was clarified by centrifugation (31 000 × g, 20 min, 4 °C), filtered through a 0.45 μm filter, and applied to a Ni²⁺-charged His·bind resin (Novagen). Following treatment with binding buffer (25 mL) and wash buffer (15 mL; 60 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.9), the His₆-tagged ODCase was eluted with elution buffer (8 mL; 5 mM UMP, 100 mM EDTA, 300 mM NaCl, 20 mM Tris base, 10% v/v glycerol, pH 7.9). The enzyme was then dialyzed against 3 × 500 mL of storage buffer (10% v/v glycerol, 30 mM Tris-Cl, 250 mM NaCl, 2 mM DTT, pH 8.0) overnight. The purified protein (8.0 mL) was stored at –80 °C. The typical yield of protein was 2.2 mg from 1.0 L of culture (Figure 1S).
Enzyme assay. The decarboxylation of OMP to UMP catalyzed by ODCase was monitored spectrophotometrically at 279 nm ($\Delta\varepsilon_{279} = 2.4 \pm 0.2$ mM$^{-1}$ cm$^{-1}$) using a Hewlett-Packard UV-Vis 8453 spectrophotometer. Assays were conducted in a quartz cuvette with a 10 mm light path at 25 °C. Reactions (1 mL total volume) were initiated by addition of ODCase (20 µL, to give a final total enzyme concentration equal to 11.0 ± 0.7 nM) to a solution of OMP (10, 20, 30, 50, 75 and 100 µM) in assay buffer (30 mM Tris-Cl, 250 mM NaCl, pH 8.0). Initial velocity data were fit to equation 1 using non-linear regression analysis and the GraphPad Prism software (v 4.00, GraphPad Software, San Diego, California). The Michaelis-Menten plot obtained from the data and the corresponding values for the kinetic parameters are shown in Figure 2S.

We found that in the presence of the 6-phosphonate analogue and at low concentrations of OMP, it was difficult to obtain reliable initial velocities using the spectrophotometric assay. To overcome this problem, we utilized a complete progress curve method similar to that reported by Porter et al. for determination of the steady-state kinetic parameters of yeast ODCase.
progress curves (Figure 3S) for the decarboxylation of OMP (2.0 µM) were followed for 5 min in the presence of the 6-phosphonate inhibitor 10 (0, 10, 20, 30, 40, 50, and 60 µM) under conditions where [S] < $K_m/7$ (equation 2). The data were then fit to the first-order decay equation (3) to give apparent $V_{\text{max}}/K_m$ values which were then re-plotted in their reciprocal form to determine the inhibition constant $K_i$ value using equation 4 (having verified that the inhibition mode was competitive as shown in Figure 2).

Figure 2S. Representative Michaelis-Menten plot (with no inhibitor) showing the dependence of initial velocities ($v_i$) on the concentration of substrate (OMP). The corresponding steady-state kinetic parameters obtained from fitting the data to equation 1 are $K_m = 14 \pm 1$ mM, $V_{\text{max}} = 0.225 \pm 0.005$ µM s$^{-1}$. The assay was conducted with [E] = 11.0 ± 0.7 nM, yielding $k_{\text{cat}} = 20 \pm 1$ s$^{-1}$ and $k_{\text{cat}}/K_m = (1.44 \pm 0.15) \times 10^6$ M$^{-1}$ s$^{-1}$.

$$v_i = \frac{d[S]}{dt} = -\frac{V_{\text{max}} [S]}{K_m + [S]}$$  \hspace{1cm} (1)

$$v_i = \frac{d[S]}{dt} = -\frac{V_{\text{max}} [S]}{K_m} \quad \text{for } [S] \ll K_m$$  \hspace{1cm} (2)

$$[P]_t = [P]_o \left(1 - e^{-\frac{V_{\text{max}} t}{K_m}}\right)$$  \hspace{1cm} (3)
Phosphonate Inhibitor of OMP Decarboxylase

Thirumalairajan, Mahaney, & Bearne

Supplementary Information

\[
\left( \frac{K_m}{V_{max}} \right)^{app} = \frac{K_m}{V_{max}} + \frac{K_m}{V_{max}} \frac{[I]}{K_i}
\]  

(4)

**Figure 3S.** Representative product curves obtained in the presence of varying concentrations of compound 10. The data shown were fit to equation 3 to obtain the values of \( \frac{V_{max}}{K_m} \) (see Figure 2). The concentrations of 10 were 0 \( \mu M \) (○), 10 \( \mu M \) (△), 20 \( \mu M \) (□), 30 \( \mu M \) (◇), 40 \( \mu M \) (●), 50 \( \mu M \) (●), and 60 \( \mu M \) (▲).

Reversed-phase HPLC. For HPLC analyses, a Waters 510 pump and 610 controller were used for solvent delivery. Injections were made using a Rheodyne 7725i sample injector fitted with a 200-\( \mu L \) injection loop. Analytes were detected by monitoring the absorbance at 260 nm using a Waters 486 absorbance detector. A Restek column (Ultra IBD, 5.0 \( \mu m \), 250 × 4.6 mm) was used to separate nucleotides after quenching of the reaction as described below. ODCase was assayed by detecting uridine monophosphate (UMP) formed after the enzymatic decarboxylation of orotidine monophosphate (OMP). The assay mixture (1.0 mL) contained 5, 10, 20, or 40 \( \mu M \) of OMP in assay buffer (Tris-Cl buffer, 30 mM, pH 8.0, containing 240 mM NaCl), 13.3 \( \mu L \) of enzyme (final [E]_T = 11 nm), and compound 10 (100 \( \mu M \)) when applicable. After incubating OMP (and/or compound 10) without the enzyme at 25 °C for 2 min, the reaction was initiated by the addition of the enzyme and was immediately divided into 4 × 230 \( \mu L \) aliquots. The reaction
was quenched by the addition of 84 µL of an ice-cold solution of 1.5 M HClO₄ after 2, 5, 9, and 14 min, and cooled on ice for 0.5 h. The pH of the reaction mixture was then adjusted to ~7.0 by the addition of an ice-cold solution of 6.0 M KOH and the reaction mixture was cooled on ice for an additional 0.5 h. The potassium perchlorate that precipitated from the reaction mixture was removed by centrifugation (14 000 × g, 10 min). The supernatant was then filtered through a 0.25 µm filter (Chromspec, ON, Canada) and analyzed immediately or stored at −20 °C (for a maximum of 2 d) prior to HPLC analysis. A sample of 200 µL of the reaction mixture was loaded onto the column and the components (unreacted OMP, UMP and/or inhibitor 10) in the reaction mixture were separated by isocratic elution with ammonium acetate buffer (20 mM, pH 5.8) at a flow rate of 1.0 mL/min. Retention times (tᵣ) of OMP, UMP, and the inhibitor 10 are as shown in Figures 4S and 5S. The amount of UMP produced from the enzymatic reaction was measured by integrating the area under the peak using PeakSimple (ver 3.29). Analyses of the data were then performed using GraphPad Prism (v 4.00, GraphPad Software, San Diego, California).

**Figure 4S**: Typical elution profile for a reaction mixture (40 µM OMP; tᵣ = 3.03 min) and the corresponding amount of UMP (tᵣ = 3.98 min) formed during the enzymatic reaction in the absence of inhibitor. The reaction was quenched after 14 min.
**Figure 5S:** Elution profile of inhibitor 10 ($t_r = 2.82$ min) after treatment with *E. coli* ODCase (11 nM) using the experimental conditions described above. The small peak at $t_r = 3.58$ min is an artifact that arises when nucleotides (OMP, UMP, or 10) are chromatographed with the elution system. Compound 10 is not a substrate for the enzyme.

<table>
<thead>
<tr>
<th>inhibitor</th>
<th>$K_i$ (µM)</th>
<th>ODCase source</th>
<th>$pK_a$</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMP (substrate)</td>
<td>0.7 ($K_m$) 14 ($K_a$)</td>
<td>Sc</td>
<td>2.4$^d$</td>
<td>7,8</td>
</tr>
<tr>
<td>pyrazofurin 5'-MP</td>
<td>0.005</td>
<td>Sc</td>
<td>6.6$^e$</td>
<td>9</td>
</tr>
<tr>
<td>6-azauridine 5'-MP</td>
<td>0.064</td>
<td>Sc</td>
<td>7.0$^f$</td>
<td>10,11</td>
</tr>
<tr>
<td>xanthosine 5'-MP</td>
<td>0.41, 130, 43</td>
<td>Sc, Mt, Pf</td>
<td>–</td>
<td>7,12</td>
</tr>
<tr>
<td>6-thiocarboxamido-UMP</td>
<td>3.5 × 10$^{-3}$</td>
<td>Sc</td>
<td>–</td>
<td>7,13</td>
</tr>
<tr>
<td>barbituric acid ribofuranoside 5'-MP (BMP)</td>
<td>8.8 × 10$^{-6}$</td>
<td>Sc</td>
<td>~2.8 - 4.5</td>
<td>11,14</td>
</tr>
<tr>
<td>6-CH$_2$NH$_2$-UMP (MAUMP$^+$)</td>
<td>≥ 15</td>
<td>Sc$'$</td>
<td>6.6</td>
<td>15</td>
</tr>
<tr>
<td>6-CH$_2$NH$_2$-UMP (MAUMP)</td>
<td>3.3</td>
<td>Sc$'$</td>
<td>–</td>
<td>15</td>
</tr>
<tr>
<td>5,6-dihydro-OMP-6-SO$_3^-$</td>
<td>20.8</td>
<td>Sc$'$</td>
<td>~1.7$^g$</td>
<td>14</td>
</tr>
<tr>
<td>5,6-dihydro-UMP-6-SO$_3^-$</td>
<td>29.2</td>
<td>Sc$'$</td>
<td>~1.7$^g$</td>
<td>14</td>
</tr>
<tr>
<td>5,6-dihydro-OMP</td>
<td>0.25</td>
<td>Sc$'$</td>
<td>3.1$^h$</td>
<td>7,16</td>
</tr>
<tr>
<td>6-NH$_2$-UMP</td>
<td>0.84, 2.1</td>
<td>Mt, Pf</td>
<td>–</td>
<td>17</td>
</tr>
<tr>
<td>6-PO$_3$H$_2$-UMP (10)</td>
<td>25.8</td>
<td>Ec</td>
<td>~6.9$^i$</td>
<td>this work</td>
</tr>
</tbody>
</table>

$^a$Abbreviations used: MP, 5'-monophosphate; U, uridine; O, orotidine; $^b$Organisms: Ec, *Escherichia coli*; Mt, *Methanobacterium thermoautotrophicum*; Pf, *Plasmodium falciparum*; Sc, *Saccharomyces cerevisiae*; Sc' (C155S mutant of Sc ODCase); $^c$Values are for ionizations of the indicated groups on the nucleotide base; $^d$p$K_a$ value for 6-COOH of orotic acid; $^e$Although this value is often quoted as the $pK_a$ of the 4-OH on the base, it is not clear that the authors in ref. 9 are referring to this group or how the value was obtained; $^f$p$K_a$ value for the triazine ring; $^g$p$K_a$ value for ethylsulfonic acid$^{18}$; $^h$p$K_a$ value for 6-COOH of 5,6-dihydroorotic acid; $^i$p$K_a$ value for ionization of 6-PO$_3$H$^-$ estimated using second $pK_a$ for phenylphosphonic acid adjusted to the ionic strength of the assay (0.4 M) at 25 °C.$^{19}$

### REFERENCES


NMR SPECTRA

$^1$H-NMR of 2',3'-isopropylidene uridine
$^{13}$C-NMR of 2',3'-isopropylidine uridine

Supplementary Information
$^{1}$H-NMR of 6a
Supplementary Information
$^{13}$C-NMR of 7a
Supplementary Information

**31P-NMR of 7a - coupled spectrum**

![31P-NMR of 7a - coupled spectrum](image1)

**31P-NMR of 7a - decoupled spectrum**

![31P-NMR of 7a - decoupled spectrum](image2)
1H-NMR of 6b
### Supplementary Material (ESI) for Chemical Communications

This journal is (c) The Royal Society of Chemistry 2010

### Table 1: NMR Data for Compound 8

|-----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|

### Diagram

#### 

**H-NMR of 8**

[Graph showing the NMR spectrum of compound 8]
Supplementary Information

Thirumalairajan, Mahaney, & Bearne

$^{13}$C-NMR of 8

Supplementary Material (ESI) for Chemical Communications
This journal is (c) The Royal Society of Chemistry 2010
Phosphonate Inhibitor of OMP Decarboxylase

Thirumalairajan, Mahaney, & Bearne

Supplementary Information

31P-NMR of 8 – coupled spectrum

31P-NMR of 8 – decoupled spectrum

Supplementary Material (ESI) for Chemical Communications
This journal is (c) The Royal Society of Chemistry 2010
$^{13}$C-NMR of 9
Phosphonate Inhibitor of OMP Decarboxylase

Thirumalairajan, Mahaney, & Bearne

Supplementary Information

This journal is (c) The Royal Society of Chemistry 2010

Supplementary Material (ESI) for Chemical Communications

31P-NMR of 9 – coupled spectrum

31P-NMR of 9 – decoupled spectrum
Supplementary Information
Supplementary Material (ESI) for Chemical Communications
This journal is (c) The Royal Society of Chemistry 2010

\[ ^{13}\text{C-NMR of 10} \]
Phosphonate Inhibitor of OMP Decarboxylase
Thirumalairajan, Mahaney, & Bearne

Supplementary Information

\[ \text{31P-NMR of 10 – coupled spectrum} \]

\[ \text{31P-NMR of 10 – decoupled spectrum} \]
**31P-NMR of 11 – coupled spectrum**

**31P-NMR of 11 – decoupled spectrum**
COSY of 11