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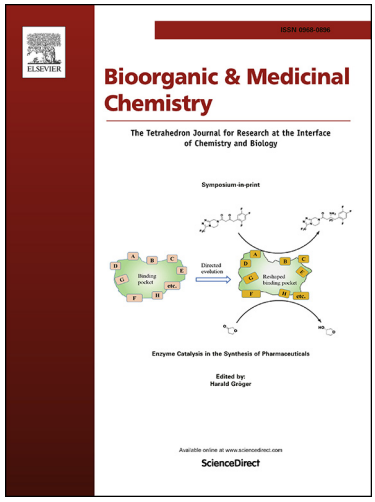

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Chemo-enzymatic synthesis of the exocyclic olefin isomer of thymidine monophosphate

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Abstract.

Exocyclic olefin variants of thymidylate (dTMP) recently have been proposed as reaction intermediates for the thymidyl biosynthesis enzymes found in many pathogenic organisms, yet synthetic reports on these materials are lacking. Here we report two strategies to prepare the exocyclic olefin isomer of dTMP, which is a putative reaction intermediate in pathogenic thymine biosynthesis and a novel nucleotide analog. Our most effective strategy involves preserving the existing glyosidic bond of thymidine and manipulating the base to generate the exocyclic methylene moiety. We also report a successful enzymatic deoxyribosylation of a non-aromatic nucleobase isomer of thymine, which provides an additional strategy to access nucleotide analogs with disrupted ring conjugation or with reduced heterocyclic bases. The strategies reported here are straightforward and extendable towards the synthesis of various pyrimidine nucleotide analogs, which could lead to compounds of value in studies of enzyme reaction mechanisms or serve as templates for rational drug design.

Introduction.

Nucleotides are essential molecules commonly referred to as the building blocks of life because they make up DNA and RNA as well as cell machinery for expression, regulation, and generation of gene products. Thus, structural analogs of nucleotides have seen a variety of uses including, but not limited to, cancer therapeutics, mechanistic bioreagents, and *in vivo* probes for metabolic studies.[1-3] Unnatural and noncanonical nucleotides have been produced through modification of the phosphate,[4-7] the sugar,[8-11] and the nucleobase[3,12-15] moieties resulting in a range of biologically relevant compounds with a variety of activities and functions.[9] The focus of this report involves nucleobase modifications that result in a new structural variant of the thymidyl moiety. In the past nucleobase analogs that mimic thymidyl moieties have produced antiviral agents (e.g., trifluorothymidine[16,17]), antitumor agents (e.g., 5-F-uridine[18,19]), and biological flourophores (e.g., 5-aminoallyluridine[20]). These past examples showcase the importance of modifications of this pyrimidine nucleotide in particular, and the targeting of thymidyl biological processes in general.

Thymine is unique among the DNA nucleosides. Deoxyguanosine, deoxyadenosine, and deoxycytosine can be produced directly from their respective ribosides through the action of ribonucleotide reductases, but thymidyl moieties must be produced *de novo* by methylation of uridine.[21] Several enzymes catalyze this reductive methylation, including thymidylate synthase (TSase),[22] flavin-dependent thymidylate synthase (FDTS),[23] and methylenetetrahydrofolate-tRNA-(uracil-5-)-methyltransferase (TrmFO),[24] to provide the thymidine that is essential for DNA production and cell proliferation.[22] As a result, thymidyl biosynthetic enzymes are often the target of chemotherapeutic agents[19] and they have shown potential as antibacterial[25] and antiviral[26] drug targets as well. However, while the well-studied "classical" thymidylate synthase (TSase) has been the focus of cancer drug development (e.g., 5-F-dU together with

leucovorin), less is known regarding the inhibition of alternative thymidyl biosynthesis enzymes, which limits the development of antibacterial and antiviral compounds that target these enzymes.[23] For example, the enzyme FDTS, which is present in several important human pathogens, functions by a remarkably different chemical mechanism from classical TSase. At this time there are no known inhibitors that selectively target FDTS.[27] Compounds that mimic the unique intermediates for this alternative thymidylate biosynthesis may advance our ability to exploit this attractive target.

All of the known biosynthetic pathways that lead to thymidylic acid (**1**) by reductive methylation, whether catalyzed by FDTS (from **2**) or by TRMFO or TSase (from **3**), have in common an exoxyclic methylene group at the C5-C7 position of the pyrimidine ring (Figure 1). The existence of exocylic methylene intermediates has been supported by nucleophilic trapping experiments which disrupt the enzymatic turnover and allow isolation of derivatives of the reaction intermediates.[25,28,29] Many of these putative intermediate species can only be hypothesized based on these chemical trapping experiments along with the known reactivity of an α,β-unsaturated system, and therefore direct access to this analog of dTMP would greatly facilitate mechanistic study of thymidyl biosynthesis.[29] Exocyclic olefin analogs of thymidine have appeared in studies by Greenberg and his group (e.g. **4**), but all the currently known compounds are also substituted at the C-6 position. Nevertheless, these doubly-modified thymidine analogues have been used to demonstrate the formation of interstrand cross-linking and biocongugates in RNA and DNA via a mechanism involving exocyclic methylene-modified thymidines.[12,13,30,31] Thus there has been substantial interest in designing synthetic routes that can generate modified nucleotides,[9,32] and one modified only by transposition of the olefin to an exocyclic position (i.e. compound **2)** would be especially valuable in studies of enzyme

mechanism(s). Unfortunately, due to the various functional groups present in the starting materials, formation of an exocylic methylene moiety is challenging and traditional reactions proceeding by installation and removal of orthogonal blocking groups ultimately impacts synthetic feasibility.[9]

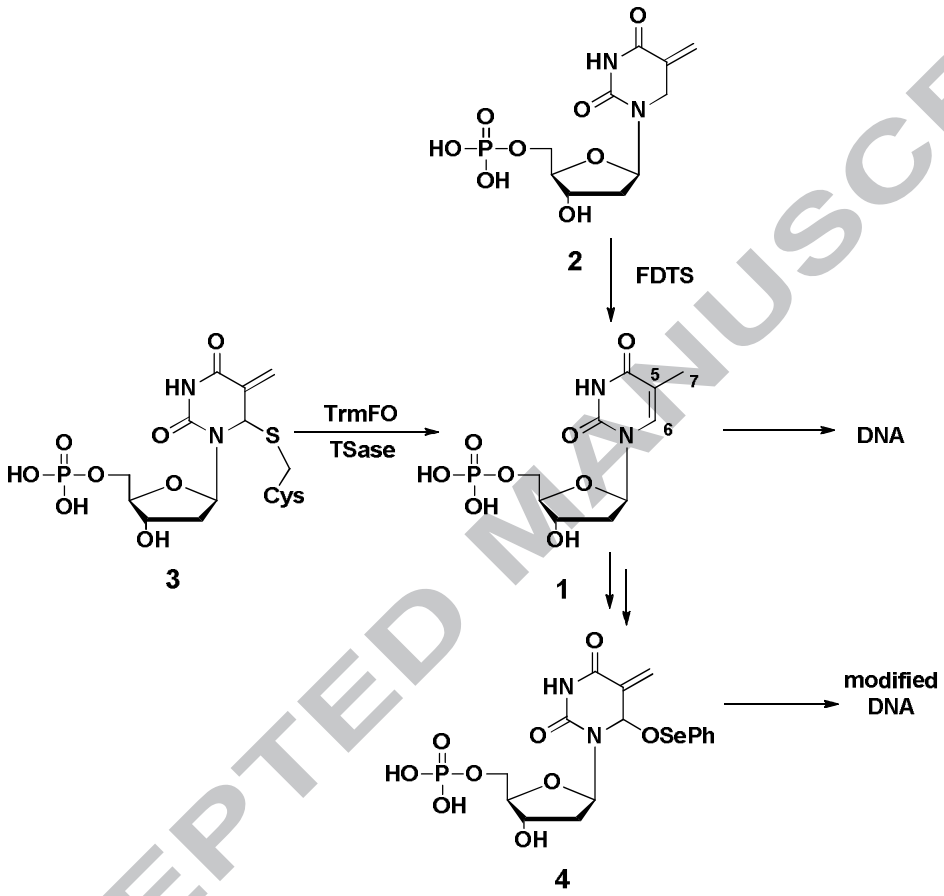


Figure 1: Thymidylic acid and some olefin analogues of biological significance.

To the best of our knowledge, only transient olefin isomers of thymidine or thymidylate have been proposed,[23] occurring either as intermediates in enzyme reactions or along the pathway to DNA labeling via photolysis. Furthermore, all of these examples have a heteroatom substituent at C-6 of the pyrimidine ring and therefore have a different C-6 oxidation state than the dihydro species proposed as an intermediate during alternative thymidylate biosynthesis (i.e. compound **2**).[23,33] In 1973 Klötzer[34] reported the synthesis of an exocyclic methylene nucleobase

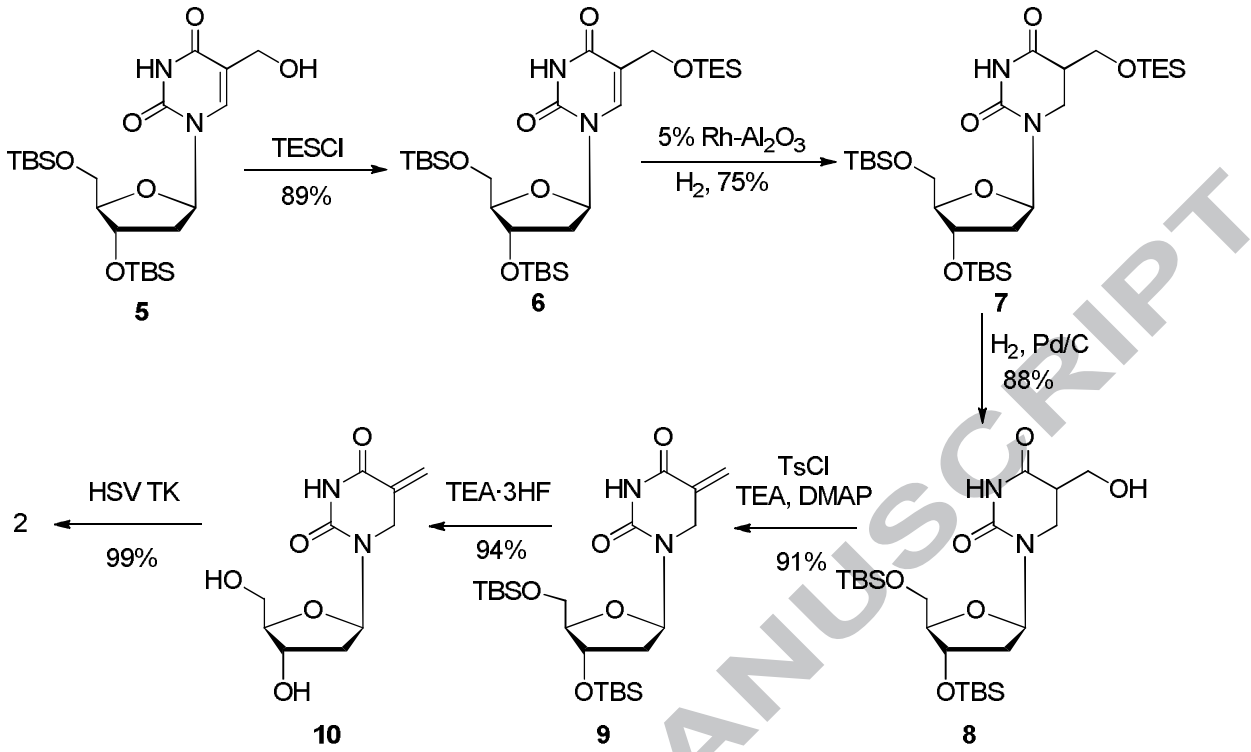
isomer of thymine, but synthesis of the exocyclic methylene isomer of thymidine or thymidylate has not been reported. Herein we present two different synthetic routes to achieve the unique C-5 isomer of dTMP (**1**) bearing an exocyclic olefin. This isomer has been proposed[25,29,35,36] as an intermediate during the FDTS catalyzed reaction and therefore represents an important structural motif in pathogenic dTMP biosynthesis. We also report on the chemical and pH stability of this compound, along with derivatization at the C-7 methylene terminus that results in novel nucleophilic addition products and may serve as a possible gateway to novel compounds with antibacterial or antiviral activities.[23] Finally, the reactivity reported herein suggests that compound **2** may have potential use in reaction with nucleophilic residues of thymidyl binding proteins or in DNA.

Results and Discussion.

Two complementary synthetic approaches have been developed to prepare the target compound **2**. Although the approaches are orthogonal in strategy, both afford the exocyclic C-5 olefin isomer of thymidine (**10**) and then follow the same phosphorylation pathway via an enzymatic kinase. Exploration of these two orthogonal synthetic strategies paves the way for future synthetic modifications that can easily afford novel isotopic labeling patterns in compound **2**.

The first approach began with thymidine and applied the general synthetic strategy of functionalizing C-7, followed by dearomatization via reduction, and finally an elimination reaction to create the exocyclic methylene group and generate compound **2** (Scheme 1). More specifically, the protected nucleoside **5** was prepared from commercial thymidine using a previously published procedure.[37] Subsequent protection of the C-7 primary hydroxyl group of compound **5** was required to prevent side products during the reduction. Introduction of the

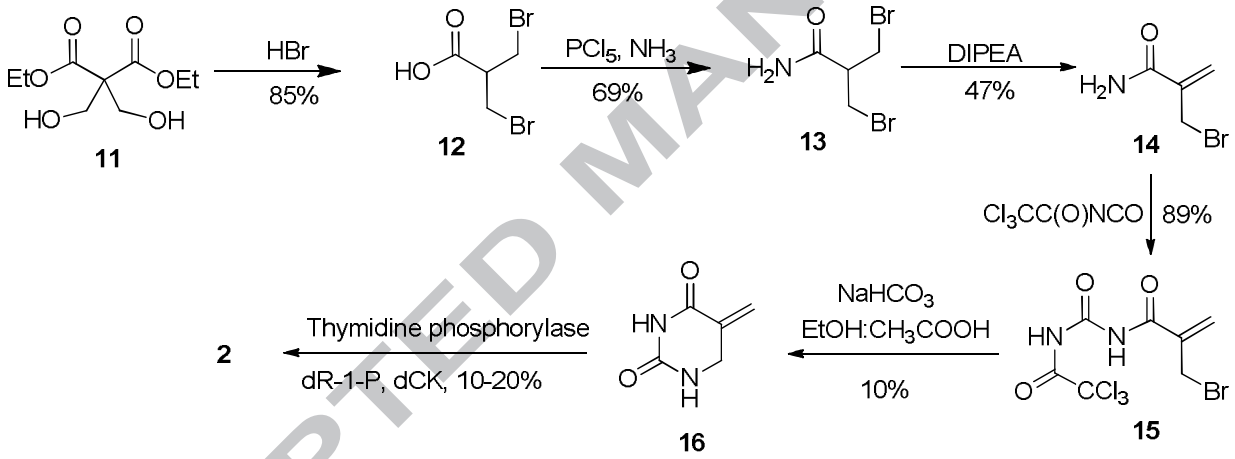
triethylsilyl group (**6**) allowed selective deprotection later in the sequence, even in the presence of two TBDMS groups. Reduction of compound **6** gave a mixture of diastereomers (**7**), of little long-term consequence given the intent to generate the exocyclic olefin. This mixture was then deprotected selectively at C-7 to obtain compound **8**. Attempted tosylation of the primary alcohol **8** resulted in a mixture of compounds and isolation of the individual components was not pursued. Remarkably, optimized conditions allowed tosylation followed by elimination in a single flask, furnishing compound **9** in high yield (91%). Removal of the TBS groups was achieved upon treatment with TEA·3HF buffered with pyridine to prevent tautomerization of the exocyclic double bond to thymidine. Phosphorylation of compound **10** proved to be challenging with $POCl_3$[38] or $(H_3CO)_3P$[39] reagents due to varying degrees of isomerization under different reaction conditions. It is possible that strategies based on other P(III) reagents[40] still could be employed to accomplish a chemical formation of the desired monophosphate, but we turned to enzymatic methods instead. When an enzymatic reaction was attempted, phosphorylation of compound **10** was achieved by a pyrimidine kinase to obtain the desired compound **2** in high yield.[41] While isolation of compound **2** was complicated by the various components necessary for the enzymatic reaction, it was possible to isolate milligrams of material by HPLC. The NMR spectra of this product were clearly consistent with the bis(triethylammonium) salt of compound **2**. Of special significance were resonances attributable to the exocyclic methylene group at 6.22 and 5.89 ppm, both of which correlated to a carbon resonance at 126.4 ppm in an HSQC experiment, as well as those at 4.15 and 4.25 ppm, which correlated to the C-6 resonance at 41.6 ppm.



Scheme 1. Synthesis of the new thymidylate analogue **2**.

Through an orthogonal synthetic strategy, compound **2** was prepared by initial synthesis of the thymine isomer using procedures based on a known synthetic route (Scheme 2).[34] In brief, commercial diethyl bis(hydroxymethyl)malonate **11** was converted to the dibromide **12** as previously described[34] providing the desired starting material. After formation of the acid chloride, reaction with ammonia gave the amide **13**, which eliminates HBr to give the olefin **14** through a procedure slightly modified from the original synthesis. Condensation of the olefin **14** with trichloroacetyl isocyanate gives imide **15**, which then can be cyclized and deacylated to give the methylene hexahydropyrimidine dione **16** in low yield. Glycosylation of compound **16** then was accomplished by enzymatic means, through the use of commercial *E. coli* thymidine phosphorylase (subjected to anaerobic dialysis) and freshly synthesized deoxyribose-1-phosphate (dR-1-P).[42] Although it is unstable, it was advantageous to use dR-1-P directly as opposed to

generation *in situ* as often reported[43,44] because this allows one to drive the reaction with an excess of the sugar phosphate (e.g. 10-fold). The final 5'-phosphorylation with a pyrimidine kinase was performed *in situ* with glycosylation to shift the equilibrium in favor of the desired product. Overall conversion in this last step was limited by the glycosylation efficiency and typically was between 10-20%, perhaps because of substantial product inhibition from inorganic phosphate. Nevertheless it should be possible to isolate a milligram quantity from these reactions. However the earlier strategy would be more easily extended to provide larger quantities of the desired product because only one enzymatic transformation is required in that sequence.



Scheme 2. Preparation of thymidine analogue **16** and its conversion to thymidylate analogue **2**.

The synthetic strategies described above are complementary and could be employed to obtain a range of products with various isotopic labeling patterns. For example, the sequence described in Scheme 1 could be used to introduce isotopic labels at the nucleobase, while Scheme 2 is attractive for introduction of radiolabeled sugar moieties with minimal downstream workup and by-product formation.

During the course of these syntheses, compound **2** was found to be unstable under various conditions. For example, at high pH (>10) it decomposes to multiple products that were not characterized. In contrast, at 14 °C it was sufficiently stable in 0.5% formic acid in water to allow LCMS analysis but at lower pH (<5) and room temperature rearrangement of the double bond is observed and compound **2** isomerizes slowly to produce dTMP. Partial isomerization to dTMP also was observed during lyophilization of compound **2**, although this could be minimized by addition of equimolar salt (NaCl or KCl) during concentration. The use of triethylammonium bicarbonate buffer (pH 7.2) or triethylammonium acetate (pH 5.6) during HPLC purification also minimized isomerization. While conditions that allow or promote isomerization should be avoided during preparation of compound **2**, the observed isomerization serves as an additional structure proof because the dTMP formed by isomerization is indistinguishable from commercial dTMP.

It is notable that the exocyclic olefin **16** is much more stable than its glycosylated counterparts **10** and **2**. Incubation of **16** under acidic (<2) or basic (>10) conditions provided no noticeable change in structure after 24 hours. Storage of compound **16** under conditions that caused derivatization of compound **2** (*vide infra*), gave no detectable addition products. These observations are consistent with the original report on the synthesis and stability of compound **16**.[34] While mechanistic studies on the precise nature of this isomerization are beyond the scope of this report, this instability should be taken as a note of caution during preparation of compounds **10** and **2**.

Compound **2** also proved to be reactive with nucleophiles. For example, incubation of the α,β-unsaturated compound **2** in a buffer solution of tris(hydroxymethyl)aminomethane (TRIS) or in the presence of dithiothreitol (DTT), led to its disappearance. Analysis of these

reaction mixtures by LCMS revealed new materials which were 121 and 154 AMU higher in molecular weight than the starting material **2**, which suggests the addition of TRIS and DTT, respectively (cf. SI). Further analysis by MSMS revealed the parent peak for compound **2**, which also supports formation of addition products. Due to the presence of an α,β-unsaturated system, it would not be surprising if compound **2** represents a good Michael acceptor as shown in Figure **2**. The potential for addition of nucleophiles to C-7 also could represent a strategy to produce a variety of addition products leading to new nucleotide analogs based on the dihydrothymine skeleton .

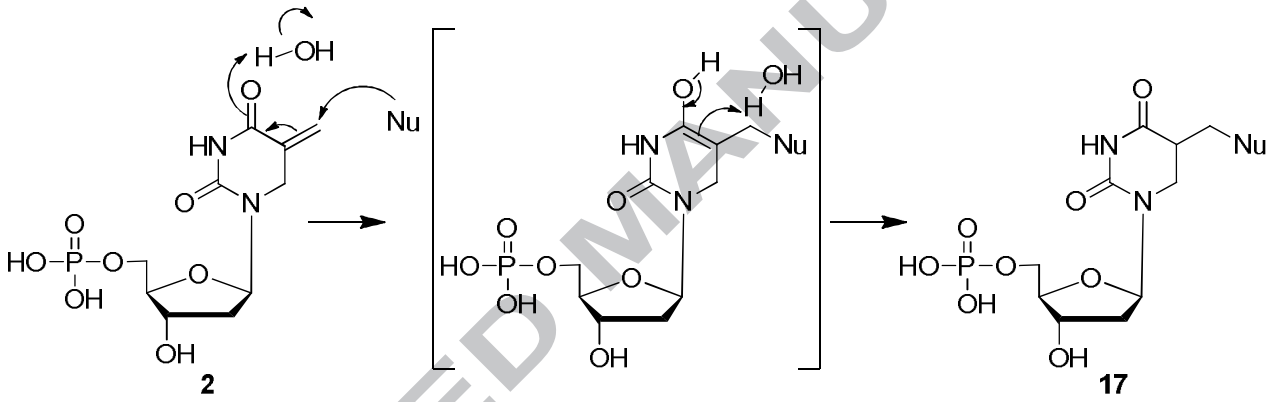


Figure 2: Nucleophilic addition to the α,β-unsaturated system **2**.

One clear application of the exocyclic olefin isomers of thymidine (**10**) and dTMP (**2**), is for direct exploration of thymidyl biosynthetic pathways. In the past, modified pyrimidines that can trap nucleophiles by reaction at C-6 have been explored as a strategy for inhibitor design and drug development targeting classical TSase. These efforts have resulted in the discovery of key chemotherapeutics.[19] They also have been crucial in rationalizing the activity of compounds like 5-F-dU, which inhibit TSase enzymes by a mechanism based on covalent addition to the especially electrophilic C-6 position of the pyrimidine. Because compounds **10** and **2** do not

have an electrophile site at C-6 they would not be expected to covalently inhibit classical TSase enzymes. However, compounds **10** and **2** closely resemble intermediates of alternative thymidyl biosynthesis pathways (e.g., FDTS), which means that these materials may be used to illuminate dTMP production in organisms relying on these alternative biosynthetic pathways. The strategies for isotopic labeling and derivatization of compound **2** enabled by the studies reported here should add to the available tools for mechanistic biochemists to differentiate classical TSases reactions from alternative thymidyl-biosynthesizing enzymes. This in turn should aid in the development of potential antibiotics with low side reactions and limited human toxicity.

Conclusions.

Two different synthetic approaches to the C-5 olefin isomer of thymidylate have been developed. For preparation of large amounts of material, the more attractive approach presented here uses commercially available thymidine as the starting material and yields compound **2** in just 6 steps. This route is scalable and the initial steps can be performed on gram scale with the final step on a milligram scale. At the same time, a complementary synthetic approach also has been developed where the key glycosylation step is enzymatic and can be performed on a milligram or sub-milligram scale, presumably with radiolabeled carbohydrates. The synthetic routes described herein are amenable to introduction of isotopic labels at a variety of sites, as well as derivatization or functionalization that provides a gateway to other novel nucleotide analogs. While the most direct application of compound **2** may be in the exploration of alternative thymidylate biosynthesis pathways, applications can be envisioned wherever the common nucleotide thymidylate is found in metabolism.

EXPERIMENTAL SECTION

General. All chemical reagents and solvents were obtained from commercial suppliers except α-hydroxythymidine (**5**),[37] 3-bromo-2-(bromomethyl)-propanoic acid (**12**),[34] and deoxyribose-1-phosphate,[42] which were prepared by known procedures. Reactions in nonaqueous solvents were performed under an argon atmosphere and monitored by analytical thin-layer chromatography on plates coated with 0.25 mm silica gel 60 F254. The TLC plates were visualized under UV light at 254 nm. All ^{1}H NMR spectra were obtained on 300, 500, or 600 MHz instruments with chemical shifts reported in ppm referenced to residual solvent in $CDCl_3$ (^{1}H, 7.26 ppm; ^{13}C NMR; 77.0 ppm), $(CD_3)_2SO$ (^{1}H, 2.50 ppm; ^{13}C NMR; 39.5 ppm), CD_3OD (^{1}H, 3.31 ppm; ^{13}C NMR; 49.0 ppm), and D_2O (^{1}H, 4.80 ppm). High-resolution mass spectra were obtained with a QTOF mass spectrometer and reported as *m/z*. LCMS traces were recorded on a Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7µm) [flow rate = 0.2 mL/min]; Line A, 0.5% formic acid and Line B, acetonitrile; run time 24 min.

3',5'-Di-*t*-butyldimethylsilyl-7-triethylsilyl-2′-deoxyuridine (6). In a round bottom flask, the primary alcohol **5**[37] (1.00 g, 2.06 mmol, 1.0 eq), imidazole (200 mg, 2.94 mmol, 1.5 eq), and TESCl (370 mg, 2.45 mmol, 1.2 eq) were dissolved in DMF (10 mL) and the solution was stirred at rt overnight. The reaction was quenched by addition of water and extracted with EtOAc (2 x 300 mL). The combined organic phase was washed with water (3 x 300 mL), dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (hexane/EtOAc; 9:1 to 4:1) to yield compound **6** (1.10 g, 89%) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.07–0.08 (m, 12H), 0.63–0.71 (m, 6H), 0.88–0.93 (m, 18H), 0.95–1.02 (m, 9H), 1.98–2.07 (m, 1H), 2.28–2.36 (m, 1H), 3.64–3.70 (m, 1H), 3.75–3.81 (m, 1H), 3.94–4.00 (m, 1H), 4.37–4.41 (m, 1H), 4.43–4.52 (m, 2H), 6.24–6.35 (m, 1H),

7.50–7.56 (m, 1H), 8.57–8.68 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ −5.4 (2C), −4.7, −4.8, 4.4 (3C), 6.8 (3C), 18.0, 18.4, 25.7 (3C), 25.9 (3C), 40.5, 57.7, 63.3, 72.6, 85.4, 87.8, 114.3, 136.0, 150.0, 162.1; HRMS (ESI-QTOF) calcd for $C_{28}H_{56}N_2O_6Si_3Na$ $(M + Na)^+$ 623.3344, found, 623.3349.

3',5'-Di-*t*-butyldimethylsilyl-5,6-dihydro-7-triethylsilyl-2′-deoxyuridine (7). Compound **6** (1.60 g, 2.66 mmol) was dissolved in a mixture of methanol (40 mL) and water (8 mL). To this solution was added the solid catalyst (Rh/Al_2O_3, 1.5 g, 5%) and the mixture was subjected to hydrogenation at rt and 170 psi for 18 h. The solution then was filtered and the filtrate was concentrated *in vacuo.* The residue was purified by silica gel column chromatography (hexane /EtOAc; 9:1 to 7:3) to yield compound **7** (1.20 g, 75%) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.07–0.08 (m, 12H), 0.59–0.64 (m, 6H), 0.90–0.91 (m, 18H), 0.94–0.98 (m, 9H), 1.97–2.02 (m, 2H), 2.66–2.74 (m, 1H), 3.40–3.47 (m, 2H), 3.59–3.65 (m, 1H), 3.68–3.74 (m, 1H), 3.77–3.80 (m, 1H), 3.84–3.89 (m, 1H), 3.97–4.03 (m, 1H), 4.33–4.39 (m, 1H), 6.27–6.31 (t, 1H), 7.64–7.73 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ −5.4 (2C), −4.8 (2C), 4.3 (3C), 6.7 (3C), 18.0, 18.4, 25.8 (3C), 25.9(3C), 37.3, 38.7, 43.4, 59.8, 63.1, 72.1, 83.8, 86.2, 152.4, 170.0; HRMS (ESI-QTOF) calcd for $C_{28}H_{58}N_2O_6Si_3Na$ $(M + Na)^+$, 625.3500, found 625.3514.

3',5'-Di-*t*-butyldimethylsilyl-5,6-dihydro-5-hydroxymethyl-2′-deoxyuridine (8).

Compound **7** (1.0 g, 1.66 mmol) was dissolved in anhydrous methanol (15 mL). To this solution was added solid Pd/C (~100 mg, 10%) and the mixture was stirred at rt under a hydrogen atmosphere for 20 minutes. The solution then was filtered through a celite bed and the filtrate was concentrated *in vacuo.* The initial product was purified by silica gel column chromatography (hexane/EtOAc; 7:3 to 1:1) to yield the primary alcohol **8** (710 mg, 88%) as a colorless oil: ^{1}H NMR (500 MHz, MeOD) δ 0.10–0.15 (m, 12H), 0.94 (d, J =3.4 Hz, 18H), 1.94 (ddd, *J* = 13.2,

6.0, 2.5 Hz, 1H), 2.14–2.22 (m, 1H), 2.70–2.78 (m, 1H), 3.46–3.56 (m, 2H), 3.68–3.78 (m, 2H), 3.78–3.87 (m, 3H), 4.39–4.48 (m, 1H), 6.27–6.30 (dd, J = 8.6, 5.9 Hz, 1H); ^{13}C NMR (125 MHz, MeOD) δ −8.2, −8.1, −7.4, −7.3, 16.0, 16.4, 23.4 (3C), 23.6 (3C), 35.0, 36.6, 41.5, 57.4, 61.7, 71.2, 82.4, 85.1, 152.0, 170.1; HRMS (ESI-QTOF) calcd for $C_{22}H_{44}N_2O_6Si_2Na$ $(M + Na)^+$ 511.2636, found 511.2636.

3',5'-Di-*t*-butyldimethylsilyl-5-methylene-6-hydro-2′-deoxyuridine (9). In a round bottom flask the primary alcohol **8** (710 mg, 1.45 mmol, 1.0 eq), tosyl chloride (555 mg 2.91 mmol, 2 eq), triethylamine (294 mg, 2.91 mmol, 2 eq) and a catalytic amount of DMAP were dissolved in CH_2Cl_2 (10 mL) and the resulting solution was stirred at room temperature overnight. The reaction mixture then was quenched by addition of water and extracted with EtOAc (3 x 50 mL). The combined organic phase was washed with water (2 x 50 mL), dried (Na_2SO_4), and filtered, and the filtrate was concentrated *in vacuo*. The initial product was purified by silica gel column chromatography (hexane/EtOAc, 9:1 to 7:3) to yield compound **9** (622 mg, 91%) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.09–0.11 (m, 12H), 0.91–0.94 (m, 18H), 1.95–2.01 (m, 1H), 2.02–2.09 (m, 1H), 3.72–3.79 (m, 2H), 3.82–3.84 (m, 1H), 3.97–4.00 (d, 1H), 4.28–4.31 (d, 1H), 4.39–4.43 (m, 1H), 5.65 (s, 1H), 6.32–6.35 (t, 1H), 6.39 (s, 1H) 7.50 (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ −5.5, −5.4, −4.8, −4.6, 18.0, 18.4, 25.8 (3C), 25.9 (3C), 37.3, 41.6, 63.0, 72.0, 83.9, 86.5, 124.7, 131.4, 152.00, 163.0; HRMS (ESI-QTOF) calcd for $C_{22}H_{42}N_2O_5Si_2Na$ $(M + Na)^+$ 493.2530, found 493.2531.

5-Methylene-6-hydro-2′-deoxyuridine (10). In a plastic vial, compound **9** (100 mg, 0.21 mmol) was dissolved in pyridine (1.5 mL) and the solution was cooled to 0 °C. To this cold solution, TEA·3HF (0.42 mmol, 2 eq) was added dropwise and the reaction was stirred for 5 h. The reaction mixture then was diluted with pyridine (5 mL) and concentrated *in vacuo.* The

initial product was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 9:1 to 8.5:1.5) to yield compound **10** (48 mg, 94%) as a white solid: ^{1}H NMR (600 MHz, D_2O) δ 2.04–2.11 (m, 1H), 2.26–2.34 (m, 1H), 3.62–3.66 (m, 1H), 3.70–3.74 (m, 1H), 3.85 (br d, *J* = 5.5 Hz, 1H), 4.14– 4.23 (m, 2H), 4.32–4.35 (m, 1H), 5.81–5.84 (m, 1H), 6.20–6.24 (m, 1H), 6.26–6.29 (m, 1H); ^{13}C NMR (126 MHz, D_2O) δ 35.0, 41.8, 61.6, 70.8, 84.2, 85.3, 126.4, 130.5, 153.8, 165.9; HRMS (ESI-QTOF) calcd for $C_{10}H_{14}N_2O_5Na$ $(M + Na)^+$ 265.0800, found 265.0796. DEPT135 and HSQC data are included in the SI.

5′-Monophospho-5-methylene-6-hydro-2′-deoxyuridine (2). Method A. HSV Thymidine kinase (200 nM) was incubated at 34 °C in the presence of compound **10** (18 mg, 18.6 mM in 4 mL final volume), 20 mM HEPES (pH 7.8), 3 mM $MgCl_2$, 3 mM KCl, 0.8 mM ATP, 59 mM creatine phosphate and a trace amount of creatine phosphokinase (CPK). The reaction was done on a 4 ml scale. After 20 h, the reaction mixture was filtered through Amicon Ultra-4 4 mL centrifugal filters (MWCO 10 KDa) at 4°C. The product was purified by HPLC (semi-prep column) using a linear gradient of MeOH (5-100%) in 4 mM TEAB pH 6.8 at 3.2 ml/min. The product peak was detected spectrophotometrically at 210, 240, 260, and 290 nm, manually collected, and the center cut fractions were combined to give ~3 mg of the pure product: ^{1}H NMR (500 MHz, D_2O) δ 1.97–2.02 (m, 1H), 2.28–2.34 (m, 1H), 3.76–3.78 (t, 1H), 3.90–3.91 (m, 1H), 4.15 (d, 1H, *J* = 15.0), 4.25 (d, 1H, *J* = 15.0), 4.41–4.44 (m, 1H), 5.89 (s, 1H), 6.18–6.31 (m, 1H), 6.22 (s,1H); ^{31}P NMR (500 MHz, D_2O) δ 3.88; HRMS (ESI-QTOF) calcd for $C_{10}H_{15}N2O_8P$ $(M – H)^-$ 321.0488, found 321.0486. The COSY and HSQC data, and LCMS data, are included in the SI.

Method B: In a plastic vial, bis(cyclohexylammonium) deoxyribose-1-phosphate (21 mg, 0.05 mmol) and compound **16** (1.5 mg, 0.012 mmol) were added to a degassed buffer (5 mL, 20 mM

HEPES, 100 mM KCL, 5 mM $MgCl_2$). To this mixture 500 units of previously dialyzed commercial thymidine phosphorylase (TP) was added and the solution was incubated at rt for 20 h. Conversion to the thymidine analogue **10** was monitored by HPLC. When >20% conversion was observed, 12.5 mg of ATP were added followed by addition of 500 ng of mutant dCK (≥ 50 units). The resulting mixture was incubated for 20-48 h at rt until quantitative conversion of the nucleoside **10** to the nucleotide monophosphate **2** was observed. The reaction mixture was filtered through a 10,000 MWCO Millipore membrane prior to HPLC purification.

3-Bromo-2-(bromomethyl)propionamide (13).[34] Through a modification of the reported procedure, compound **12** (46 g, 190 mmol) was dissolved in anhydrous benzene (300 mL) and the solution was cooled to 0 °C. After PCl_5 (48 g, 230 mmol) was added to the cold solution in four portions (to avoid a vigorous reaction), the reaction mixture allowed to warm to rt slowly, and then heated at reflux for 2 h. The solvent was removed *in vacuo*, petroleum ether (60-80 °C) was added to the resulting residue, and the mixture was filtered. Anhydrous NH_3 was bubbled through the filtrate to give a white precipitate, which was isolated by filtration, washed with water, and dried under vacuum desiccation to give compound **13** (31.8 g, 69%): ^{1}H NMR (300 MHz, $D_3CS(O)CD_3$) δ 3.0–3.12 (m 1H) 3.5–3.7 (m, 4H) 7.32 (s, 1H) 7.71 (s, 1H).

2-Bromomethylacrylamide (14).[34] 1,4-Dioxane (27 mL) was added to a 250 mL Schlenk flask, diisopropylethylamine (6.5 mL) was added, and the mixture was degassed by several freeze-pump-thaw cycles. The resulting solution was transferred by cannula to a 100 mL Schlenk flask that contained the dibromide **13** (8.0 g, 33 mmol) and hydroquinone (30 mg, 0.27 mmol). The resulting solution was stirred for 9 hours at 50–60 °C. After concentration by vacuum distillation, ice water (5 mL) and HCl (1 N, 1 drop) were added along with ethyl acetate (20 mL). This solution was extracted with ethyl acetate and the combined extracts were concentrated *in*

vacuo to give the acrylamide **14** (2.5 g, 47%) as a cream-colored solid: ^{1}H NMR (300 MHz, $(CD_3)_2SO$) δ 4.75 (s, 2H), 5.85 (s, 1H), 5.92 (s, 1H), 7.31 (s, 1H), 7.73 (s, 1H); HRMS (ESI-QTOF) calcd for C_4H_6BrNO 162.9633 (M^+), found (EI+) 162.9633.

***N*-Trichloracetyl-*N*'-2-bromomethacroyl-urea (15).**[34] Compound **14** (1.76 g, 11 mmol) and benzene (38 mL) were added to a 250 mL Schlenk flask, followed by the addition of trichloroacetyl isocyanate (2.0 g, 11 mmol). This solution was heated at reflux for one h, allowed to cool, and gravity filtered, and the filtrate was concentrated to ~ 11 mL *in vacuo*. Dropwise addition of petroleum ether (40–60 °C) yielded compound **15** as a white crystalline solid (3.32 g, 89%): ^{1}H NMR (300 MHz, $D_3CS(O)CD_3$) δ 4.42 (s, 1H), 4.5 (s, 1H) 6.2–6.45 (m, 2H) 11.57 (s, 1H), 12.52 (s, 1H).

Dihydro-5-methylene-2,4(1*H*,3*H*)-pyrimidinedione (16).[34] A solution of $NaHCO_3$ (0.86 g, 10 mmol) in water (7.2 mL) was stirred while bromide **15** (1.5 g, 4.3 mmol) was added to produce a suspension. Ethanol (13.2 mL) was added and the reaction was heated quickly to 55-60 °C. After 2 minutes the solution was gravity filtered, the filtrate was allowed to cool to rt for 1 hour, and then concentrated *in vacuo* at 30 °C until a precipitate was observed. A minimum amount of 1N NaOH was added at 0 °C resulting in a clear solution that was filtered and then neutralized by addition of glacial acidic acid. Crystallization occurred upon dropwise addition of EtOH at 0 °C to give the initial product. Recrystallization from water and ethanol gave the desired product **16** (54 mg, 10%) as a white crystalline solid: ^{1}H NMR (300 MHz, $D_3CS(O)CD_3$) δ 3.87 (s, 2H) 5.53 (s, 1H) 5.97 (s, 1H) 7.41 (s, 1H) 10.0 (s, 1H); HRMS (ESI-QTOF) calcd for $C_5H_6N_2O_2$ (M^+) 126.0430, found (EI+) 126.0432.

Supporting Information Available: Both ^{1}H and ^{13}C NMR spectra and LCMS data. This material is available free of charge via the Internet at:

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Notes.

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