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Ground State, Intermediate, and Multivalent Nucleotide Analogue Inhibitors of Cytidine 5′-Triphosphate Synthase

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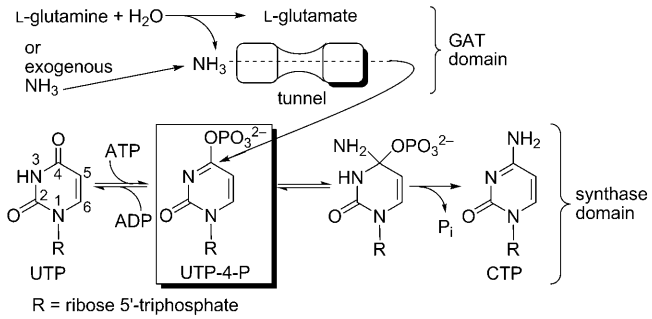
Cytidine 5′-triphosphate synthase [EC 6.3.4.2; CTPS] catalyzes the ATP-dependent formation of CTP from UTP, the final step in the de novo biosynthesis of pyrimidine nucleotides. The enzyme uses either L-glutamine (Gln) or NH_3 as the nitrogen source (Scheme 1).[1] The hydrolysis of Gln occurs in the C-terminal Gln amide transfer (GAT) domain and is promoted by the allosteric effector GTP.[2,3] The resulting nascent NH_3 is subsequently transferred to the N-terminal synthase domain via an NH_3 tunnel,[4–6] where it reacts with UTP that has been activated by ATP-dependent phosphorylation at the 4-position (i.e., UTP-4-P).[7] The enzyme exhibits positive cooperativity for ATP and UTP,[8–10] and these nucleotides act synergistically to promote tetramerization of the enzyme to its active form.[10] The product CTP is a feedback inhibitor of the enzyme[8] and plays an important role in the biosynthesis of nucleic acids, phospholipids,[11,12] and sialic acid.[13] Consequently, CTPS is a recognized target for the development of antineoplastic,[14] antiviral,[15] and antiprotozoal[16–18] agents. Although many studies have focused on delineating the regulatory properties of CTPS,[19–23] very few have focused on the development of nucleotide analogues as inhibitors of this enzyme.[3,24] 3-Deazauridine 5′-triphosphate (IC_{50} ~18 μM)[25] and cyclopentenyl cytosine (CPEC) 5′-triphosphate (IC_{50} ~6 μM)[26] are substrate and product analogues, respectively, that are the most well-studied inhibitors of CTPS. In addition, the 5′-triphosphate of 2′,2′-difluorodeoxycytidine (gemcitabine) is also believed to exert its chemotherapeutic effects, in part, through the inhibition of CTPS.[27,28] However, mutations in CTPS lead to the loss of feedback inhibition by CTP and resistance to the cytotoxic effects of these and other chemotherapy drugs.[29–37] Therefore, the development of new, potent, and selective CTPS inhibitors such as transition state/intermediate analogues or multivalent inhibitors is required.[38–40]



Scheme 1. Catalytic mechanism of CTP synthase.

Enzymes often exhibit high affinity for structural and electronic mimics of transition states or reactive intermediates generated during catalysis.[39] We rationalized that CTPS inhibitors might be developed by employing methylene or difluoromethylene phosphonate groups, or a phosphoramidate group as isosteric and/or isoelectronic replacements for the 4-phosphate group of the UTP-4-P intermediate (Scheme 1).[41–44] Alternatively, the use of multivalent ligands[38] (e.g., substrate–product analogues) is often an effective strategy to develop potent and specific enzyme inhibitors, as such inhibitors may interact with multiple binding determinants on the enzyme to afford an entropic advantage in binding.[45–48] The X-ray crystal structures of *Escherichia coli* CTPS suggest that UTP and CTP share the same binding pocket for their 5′-triphosphate groups (Figure 1).[6,49] Consequently, multivalent ligands containing binding determinants of the ribose and pyrimidine moieties of UTP, CTP, or nucleotide analogues are attractive candidates for development as CTPS inhibitors. Herein we

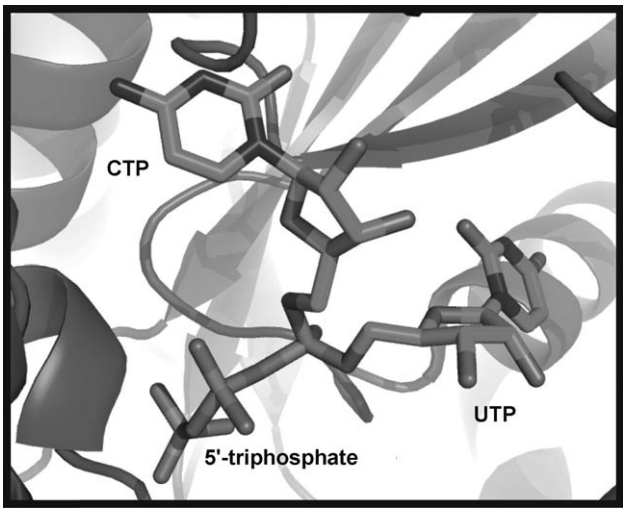


Figure 1. UTP and CTP binding sites of *E. coli* CTPS. UTP and CTP (stick representations) are shown located within the active site and share the same binding site for their 5′-triphosphate moieties as proposed by Baldwin and co-workers.[6] This figure was generated using MacPyMOL (DeLano Scientific LLC; http://www.pymol.org).

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report the results of our inhibition studies with *E. coli* CTPS and ground state and intermediate analogues[43,44,50] (**1**–**6**, Figure 2). We also report the synthesis of multivalent nucleotide analogues **7**–**10** (Figure 2) and their evaluation as inhibitors of *E. coli* CTPS. Our inhibition studies reveal that the intermediate and multivalent nucleotide analogues are indeed inhibitors of CTPS from *E. coli* and offer an excellent scaffold for further inhibitor development.

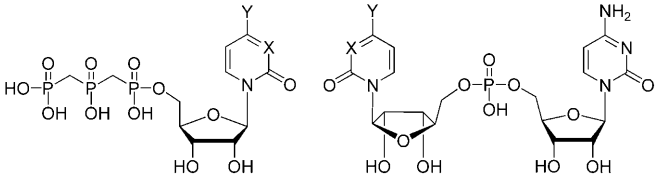
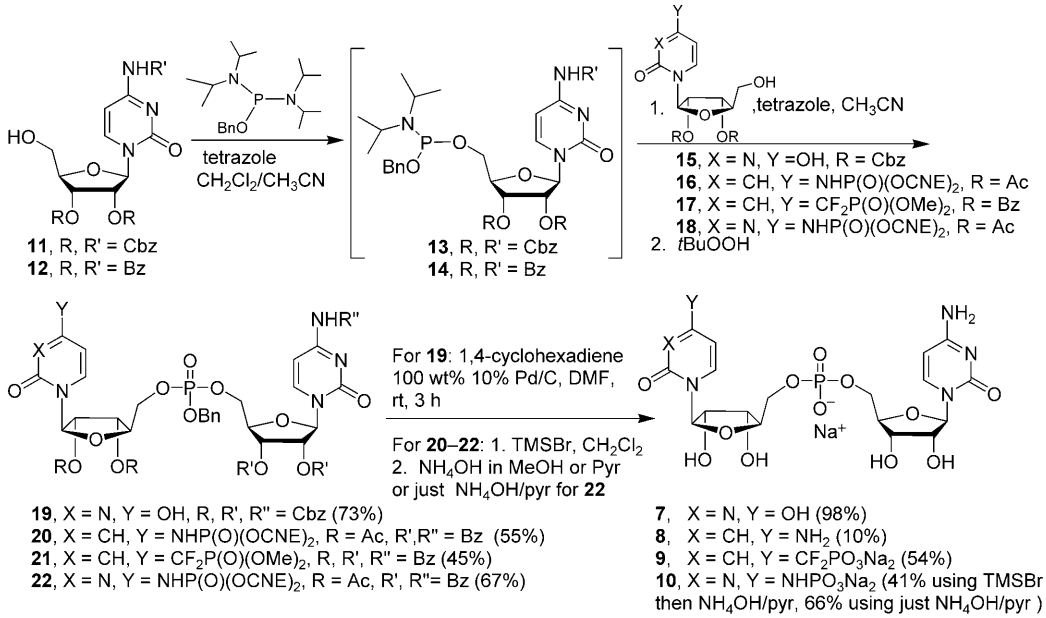


Figure 2. Structures of the ground state (**1** and **2**), intermediate (**3**–**6**), and multivalent (**7**–**10**) nucleotide analogues.

There are very few reports of pyrimidine nucleotide analogues that bear a phosphonate[43,44] or phosphoramidate[51] group on the pyrimidine moiety. The lack of such nucleotide analogues may arise, in part, from the synthetic challenges encountered when selective phosphorylation and specific solubility properties are required. Recently, however, we described the synthesis of compounds **3**–**6**, a novel class of anionic pyrimidine nucleotides.[43,44] These intermediate analogues were prepared as the 5′-bismethylene triphosphates (BMTs),[50] rather than the 5′-triphosphates, to avoid potential complications such as premature hydrolysis of the triphosphate group during synthesis.

The multivalent nucleotide analogues **7**–**10** were prepared using the procedures outlined in Scheme 2 (see Supporting Information). Nucleosides **11**[52] and **12**[53] were reacted with benzyloxybis(diisopropylamino)phosphine[54] in the presence of tetrazole to give phosphoramidites **13** and **14**. Reaction of phosphoramidite **13** with nucleoside **15**[52] and phosphoramidite **14** with nucleosides **16**,[43,44] **17** (see Supporting Information), and **18**[43,44] in the presence of tetrazole followed by oxidation of the resulting phosphites with *tert*-butylhydroperoxide gave protected dinucleotides **19**–**22** in 45–73% yield. Global deprotection of **19** using catalytic transfer hydrogenolysis gave the uridine–cytidine nucleotide **7** in 98% yield. Surprisingly, treatment of deaza compound **20** with TMSBr followed by treatment with NH_4OH-pyridine (9:1) or NH_4OH/MeOH (3:2) did not yield the desired deprotected dinucleotide bearing the phosphoramidate moiety. Instead, the dephosphorylated product **8** was the dominant product, and, after a difficult HPLC purification, we were able to isolate compound **8** albeit in only 10% yield. This is in stark contrast to the 41% yield obtained for the aza analogue **10** when compound **22** was subjected to the same reaction conditions. We later discovered that global deprotection of **22** could be achieved in an overall 66% yield by using just NH_4OH/pyridine (no TMSBr) though these conditions gave a complex mixture of products when applied to compound **20**. As we have noted with other phosphoramidate-based UTP-4-P analogues,[44] compound **10** undergoes a slow dephosphorylation at the 4-position in water. ^{1}H NMR and analytical HPLC analyses suggested that compound **10** was contaminated with 2–4% of the dephosphorylated material, which we were unable to remove. Global deprotection of compound **21** using TMSBr followed by treat-



Scheme 2. Synthesis of multivalent nucleotide analogues **7**–**10**.

ment with NH_4OH/MeOH (3:2) proceeded smoothly and gave the desired dinucleotide **9** in 54% yield.

To evaluate the intermediate and multivalent nucleotide analogues as CTPS inhibitors, we examined their ability to inhibit the NH_3-dependent CTP formation catalyzed by CTPS from *E. coli*. This approach avoids high absorbance values encountered when measuring Gln-dependent CTP formation in the presence of GTP and added nucleotide inhibitors. At present, a detailed kinetic mechanism accounting for the cooperative binding of the nucleotide substrate UTP and the inhibitors by CTPS is not available, making determination of the exact mode of inhibition difficult. Consequently, CTPS inhibition was assessed using IC_{50} values.[3] To assess the effect of substitution of the 5′-BMT for the 5′-triphosphate group on the binding of the intermediate analogues to CTPS, the inhibition of CTPS by the ground state analogues uridine 5′-BMT (**1**) and cytidine 5′-BMT (**2**) was examined. Examination of the IC_{50} values for these two compounds (Table 1) revealed that **1** is only a weak inhibitor of CTPS activity with an IC_{50} value of ~2.0 mM. This value is ~20-fold greater than the $[S]_{0.5}$ value of 99 μM for UTP.[55] Much more potent inhibition was observed with the CTP analogue **2**, with the IC_{50} value of 0.42 mM being similar to that observed for inhibition by CTP (i.e., IC_{50} = 0.30 mM; Table 2).[3] These observations suggest that the presence of the 5′-BMT group decreases the binding affinity when the pyrimidine is uridine, but not when it is cytidine. Incubation of **1** (2 mM) with CTPS in the presence of ATP and NH_4Cl gave no change in the absorbance at 291 nm over 5 min, indicating that **1** is not a substrate for CTPS. Hence, the 5′-BMT group appears to alter the binding of the UTP analogue so that it is catalytically inactive. This observation is consistent with the stringent substrate specificity of *E. coli* CTPS with respect to UTP analogues.[24]

Table 1. Inhibition of *E. coli* CTPS-catalyzed NH_3-dependent CTP formation by ground state, intermediate, and multivalent analogues **1**–**10**.

	NH_4Cl		NH_4Cl + Gln	
Inhibitor	IC_{50} [mM][a]	*n*	IC_{50} [mM][a]	*n*
1	~2.0[b]	–[c]	ND[d]	ND
2	0.42 ± 0.01	1.9 ± 0.2	0.55 ± 0.07	1.9 ± 0.5
3	0.26 ± 0.04	2.9 ± 0.1	ND	ND
4	0.13 ± 0.01	2.3 ± 0.2	0.13 ± 0.01	2.5 ± 0.1
5	0.18 ± 0.01	1.8 ± 0.1	0.25 ± 0.03	2.1 ± 0.1
6	0.17 ± 0.05	1.7 ± 0.1	0.18 ± 0.01	1.6 ± 0.2
7	0.42 ± 0.01	2.3 ± 0.1	ND	ND
8	0.19 ± 0.01	2.3 ± 0.2	ND	ND
9	0.21 ± 0.01	3.1 ± 0.2	ND	ND
10	0.13 ± 0.01	2.28 ± 0.01	ND	ND

[a] Assays conducted in the presence of either NH_4Cl (150 mM) or NH_4Cl (150 mM) and Gln (10 mM). [b] Material only available for a single determination. [c] No cooperativity observed. [d] Not determined.

Table 2. Inhibition of *E. coli* CTPS-catalyzed NH_3-dependent CTP formation by uridine, cytidine, and cytidine nucleotides.

Inhibitor	IC_{50} [mM]	*n*
uridine	3.8 ± 0.1	2.7 ± 0.2
cytidine	0.50 ± 0.01	2.1 ± 0.3
CMP	0.36 ± 0.04	1.8 ± 0.5
CDP	0.40 ± 0.05	1.9 ± 0.1
CTP[a]	0.30 ± 0.05	2.2 ± 0.1

[a] Data from reference [3].

All the intermediate analogues (**3**–**6**) that we synthesized based on the structure of the UTP-4-P intermediate inhibited CTPS with IC_{50} values in the 0.13–0.26 mM range (Table 1). Compounds **3** and **5** contain a 2-pyridinone as the base, rather than a 2-pyrimidinone, thereby mimicking the structure of 3-deaza-UTP, a known CTPS inhibitor.[25] The UTP-4-P analogue bearing a methylene phosphonate at the 4-position of the pyridinone ring (compound **3**) proved to be the weakest inhibitor. Substitution with a difluoromethylene group at the 4-position of the pyridinone ring (compound **5**) yielded slightly better inhibition as might be expected owing to the electronegative fluorine atoms decreasing the pK_a value of the phosphonate group to a value similar to that of a phosphate group, and potentially acting as H-bond acceptors.[41,42] Pyrimidinone **4** is a twofold more potent inhibitor than its pyridinone analogue **3** and exhibited the most potent inhibition of all of the intermediate analogues. Substitution of the 4-phosphonate moiety of **4** with a 4-phosphoramidate group (compound **6**) resulted in a slightly less potent, but still very effective, inhibitor.

Rapid quench and isotope partitioning experiments have revealed that Gln shifts the internal equilibrium on CTPS to favor formation of the UTP-4-P intermediate, relative to bound ATP and UTP, by a factor of 50.[56] Therefore, we examined the inhibition of CTPS activity by **2**, **4**, **5**, and **6** in the presence of NH_3 and Gln to determine whether the IC_{50} values were decreased (Table 1). Whereas the IC_{50} value for compound **2** was almost unchanged upon addition of a saturating concentration Gln (10 mM), as expected for a ground state analogue, the IC_{50} values for the intermediate analogues **4**–**6**, in the presence of Gln, were also very similar to the values determined in the absence of Gln. Hence, CTPS does not appear to recognize these compounds as intermediate analogues. This result is not surprising when one considers that the IC_{50} values for both the ground state and intermediate analogues are similar, and that uridine 5′-BMT (**1**) is not a substrate probably due to the 5′-BMT group altering the binding of the base moiety. In addition, the triphosphate group is crucial for UTP binding (cf. IC_{50} for uridine ~4 mM), but the binding of cytidine and cytidine nucleotides is not as sensitive to the degree of 5′-phosphorylation (Table 2). This suggests that the 5′-triphosphate moiety is required for specific recognition of the phosphonate and phorphoramidate compounds as true UTP-4-P analogues. However, our observation that this new class of nucleotide analogues do inhibit CTPS suggests that the presence of the methylene phosphonate group (in **4**) or the phosphoramidate group (in **6**) at the 4-position of a pyrimidine is tolerated by the enzyme.

At present, we cannot rule out that inhibition of CTPS by the intermediate analogues and the multivalent nucleotide analogues (see below) may result from an effect on the quaternary structure of the enzyme. Tetramerization of CTPS to its active form can be induced either through the synergistic

action of UTP and ATP binding, or in the presence of elevated concentrations of ATP, UTP, or CTP.[10,55,57] The ability of CTP to promote tetramerization[55,57] suggests that it would be unlikely that inhibition by the CTP analogue **2** arises from dissociation of CTPS tetramers. The similarity of the IC_{50} values for the ground state and intermediate analogues and the enzyme's apparent recognition of the intermediate analogues as ground state analogues suggest that the same might be true for compounds **3**–**6**. The fact that the Hill coefficients (*n*) for inhibition by most of the analogues range between 1.7 and 3.1 indicates that there is cooperativity in inhibitor binding. Such cooperativity could arise from the interaction between subunits within the CTPS tetramer.

All the multivalent nucleotide analogues **7**–**10** examined in this study inhibit CTPS with only about a threefold difference among all the IC_{50} values (Table 1), consistent with the notion that CTP and UTP share a mutual binding pocket on CTPS.[6,49] Interestingly, compound **7**, with cytidine and uridine linked by a 5′,5′-phosphodiester, was the weakest inhibitor. Inhibition was enhanced approximately twofold by coupling cytidine and 3-deazacytidine through a 5′,5′-phosphodiester (compound **8**). In addition, coupling of the nucleosides corresponding to the intermediate analogues **5** and **6** to cytidine with a 5′,5′-phosphodiester linkage also yielded compounds (**9** and **10**) that exhibited good inhibition of CTPS activity. Compound **10** was the best inhibitor with an IC_{50} value of 0.13 mM, approximately 2.3-fold less than the IC_{50} value for the feedback inhibitor CTP.[3]

Several informative results were obtained from these studies. First, studies with the ground state analogue **1** suggest that the BMT group is not a suitable replacement for the triphosphate group of UTP-based CTPS inhibitors. On the other hand, studies with the ground state analogue **2** suggest that such a substitution can be used for CTP-based inhibitors. Second, the intermediate analogues were indeed effective CTPS inhibitors, though it is unlikely that the inhibition is a result of their binding to CTPS in a manner similar to UTP-4-P. It was most surprising that all analogues exhibited similar IC_{50} values, despite significant structural variation among the analogues. This lack of binding discrimination may arise because the BMT moiety causes the intermediate analogues to bind in a less favorable orientation, or because the analogues are being accommodated within the CTP binding pocket and that this site exhibits some promiscuity with respect to nucleotide ligands. Such promiscuity may also explain the recently reported inhibition of CTPS by a broad range of nucleosides.[3] The triphosphate derivatives of the intermediate analogues will provide a more realistic assessment of this approach to CTPS inhibitor design, and the preparation of the 5′-triphosphate of **6** is being pursued. Finally, the results obtained with the multivalent nucleotide analogues, all of which were relatively good CTPS inhibitors, are of particular interest. Although none were highly potent inhibitors, the fact that the multivalent nucleotide analogues lack a triphosphate group, which is known to be crucial for the binding of UTP,[58] yet exhibit an affinity for CTPS similar to or better than that exhibited for UTP and CTP, leads to the conclusion that the use of multivalent nucleotide analogues is an effective strategy for the development of CTPS inhibitors. While using the 5′,5′-phosphodiester linkage simplified the synthesis of the multivalent inhibitors, additional mimicry of the 5′-triphosphate or variation of the linker length may furnish improvements in inhibitor binding, and the syntheses of such compounds are currently being pursued.

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