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# Inhibition of CTP synthase from *Escherichia coli* by xanthines and uric acids

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ABSTRACT

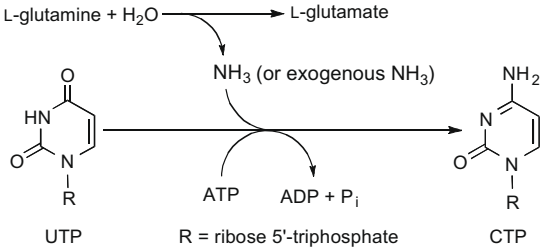
CTP synthase (CTPS) catalyzes the conversion of UTP to CTP and is a recognized target for the development of anticancer, antiviral, and antiprotozoal agents. Xanthine and related compounds inhibit CTPS activity ($IC_{50}$ = 0.16–0.58 mM). The presence of an 8-oxo function (i.e., uric acids) enhances inhibition ($IC_{50}$ = 0.060–0.121 mM). An intact purine ring with anionic character favors inhibition. In general, methylation of the purine does not significantly affect inhibition.



The glutamine amidotransferase cytidine 5-triphosphate synthase [EC 6.3.4.2; UTP–ammonia ligase (ADP-forming); CTPS] catalyzes the ATP-dependent formation of CTP from UTP using either L-glutamine (Gln) or $NH_3$ as the nitrogen source (Scheme 1).[1]

The enzyme is regulated in a complex fashion. GTP acts a positive allosteric effector for Gln-dependent CTP formation;[2,3] however, at concentrations exceeding 0.15 mM, GTP inhibits Gln-dependent CTP formation.[4] Moreover, GTP is an inhibitor of $NH_3$-dependent CTP formation at all concentrations.[4] The enzyme exhibits positive cooperativity for ATP and UTP,[5–7] and these nucleotides act synergistically to promote tetramerization of the enzyme to its active form.[7] The product CTP acts as a feedback inhibitor.[5] The human enzyme[8–10] and the yeast enzyme, encoded by the *URA7* gene,[11,12] are also regulated by phosphorylation.

The central role of CTP in the biosynthesis of nucleic acids,[13] phospholipids,[14,15] and sialic acid[16] makes CTPS an attractive target for the development of antineoplastic,[13] antiviral,[17] and antiprotozoal[18–20] agents. However, very few studies have focused on the development of nucleotide analogues as inhibitors of this enzyme.[21–23] 3-Deazauridine 5′-triphosphate ($IC_{50}$ ~18 μm)[24] and cyclopentenyl cytosine (CPEC) 5′-triphosphate ($IC_{50}$ ~6 μm)[25] are substrate and product analogues, respectively, and are the most extensively 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.[26,27] Unfortunately, mutations in CTPS lead to the loss of feedback inhibition by CTP and resistance to the cytotoxic effects




**Scheme 1.** Reactions catalyzed by CTP synthase.

of these and other chemotherapy drugs;[28–36] and CPEC treatment has been associated with cardiotoxic effects.[37] Hence, the development of new, potent, and selective CTPS inhibitors is required.

Recently, we demonstrated that the inhibition of *Escherichia coli* CTPS by GTP does not require the presence of the ribose 5′-triphosphate moiety.[22] We demonstrated that 6-thioguanine inhibits CTPS activity, suggesting that inhibition arises primarily through interactions between CTPS and the purine base. To test this hypothesis further, we examined the inhibition of CTPS by xanthine, uric acid, and their methylated derivatives. Herein, we provide the first demonstration that soluble purine derivatives are potent inhibitors of *E. coli* CTPS.

The ability of xanthines, uric acids, and analogues (Fig. 1; Sigma -Aldrich Canada, Ltd. Mississauga, ON) to inhibit both $NH_3$- and Gln-dependent CTP formation, catalyzed by CTPS from *E. coli*, was examined. Recombinant CTPS bearing an N-terminal hexahistidine tag was overexpressed in *E. coli* BL21(DE3) cells and purified using $Ni^{2+}$-affinity chromatography as described previously.[22] The

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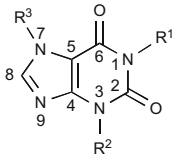
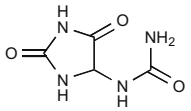
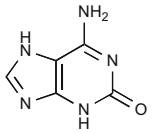


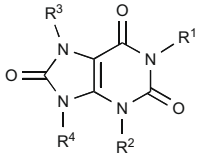
**1**, $R^1 = R^2 = R^3 = H$
**2**, $R^1 = R^2 = R^3 = CH_3$
**3**, $R^1 = R^2 = CH_3$, $R^3 = H$
**4**, $R^1 = CH_3$, $R^2 = H$, $R^3 = CH_3$
**5**, $R^1 = H$, $R^2 = R^3 = CH_3$



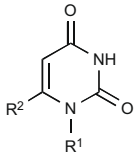
**13**, allantoin



**14**, adenine



**6**, $R^1 = R^2 = R^3 = R^4 = H$
**7**, $R^1 = CH_3$, $R^2 = R^3 = R^4 = H$
**8**, $R^1 = R^2 = CH_3$, $R^3 = R^4 = H$
**9**, $R^1 = CH_3$, $R^2 = H$, $R^3 = CH_3$, $R^4 = H$
**10**, $R^1 = H$, $R^2 = R^3 = CH_3$, $R^4 = H$
**11**, $R^1 = R^2 = R^3 = CH_3$, $R^4 = H$
**12**, $R^1 = R^2 = R^3 = R^4 = CH_3$



**15**, $R^1$ = β-D-ribose, $R^2 = H$
**16**, $R^1 = R^2 = H$
**17**, $R^1 = H$, $R^2 = CH_2CO_2H$

**Figure 1.** Structures of xanthine (**1**), uric acid (**6**), their methylated derivatives (**2**–**5** and **7**–**12**, respectively), and other analogues (**13**–**17**).

affinity tag was removed using thrombin-catalyzed cleavage and the CTPS-catalyzed conversion of UTP to CTP activity was followed using a continuous spectrophotometric assay as described previously.[22]

Xanthine (**1**) and its derivatives, differing in the location and degree of methylation (**2**–**5**), inhibited CTPS-catalyzed $NH_3$- and Gln-dependent CTP formation with $IC_{50}$ values ranging between 0.22–0.58 mM and 0.16–0.44 mM, respectively (Table 1). Fitting the kinetic data to Eq. 1 (where $v_i$ and $v_o$ are the initial velocities in the presence and absence of inhibitor, respectively, $[I]$ is the concentration of inhibitor, $K' = IC_{50}^n$, and $n$ is the Hill number) revealed that caffeine inhibited CTPS activity in a sigmoid fashion (Fig. 2). Eqs. 2, 3, and 4 describe the dependence of $K'$ on substrate concentration ($[S]$) for a competitive, uncompetitive, and noncompetitive multisite inhibitor, respectively.[38,39] Table 2 shows that $K'$ was independent of [substrate], indicating that caffeine behaves as a noncompetitive, multisite inhibitor with respect to $NH_3$, and also with respect to ATP (Fig. 2). Unfortunately, a detailed kinetic mechanism accounting for the inhibition of GTP-dependent activation and inhibition is not available,[22] making determination of the exact mode of inhibition with respect to GTP difficult. Consequently, we have relied on $IC_{50}$ values as an empirical estimate of inhibitor potency. For all compounds examined, the kinetic data were fit to Eq. 1 and $n$ >1 was observed in all cases indicating cooperativity in inhibitor binding.

$$\frac{v_i}{v_o} = \frac{K'}{K' + [I]^n} \quad (1)$$

$$K' = \alpha K_i^n + (\alpha K_i^n / K_S)[S] \quad \text{(competitive)} \quad (2)$$

$$K' = \alpha K_i^n + (\alpha K_i^n K_S)/[S] \quad \text{(uncompetitive)} \quad (3)$$

$$K' = \alpha K_i^n \quad \text{(noncompetitive)} \quad (4)$$

While xanthine and its methylated derivatives afforded only modest inhibition of CTPS activity, the presence of an 8-oxo group, as in uric acid (**6**) and its derivatives with varying degrees and positions of methylation (**7**–**12**), enhanced the inhibition ~4–7-fold with $IC_{50}$ values ranging between 0.072–0.121 mM and 0.060–0.113 mM for the inhibition of $NH_3$- and Gln-dependent CTP

**Table 1**
$IC_{50}$ Values for the inhibition of *E. coli* CTPS-catalyzed $NH_3$- and Gln-dependent CTP formation by xanthine, uric acid, and their derivatives[a]

| Inhibitor | $NH_4Cl$[b] | | Gln[c] | |
|---|---|---|---|---|
| | $IC_{50}$ (mM) | $n$ | $IC_{50}$ (mM) | $n$ |
| Xanthine (**1**), pH 8.0 | 0.37 ± 0.02 | 2.9 ± 0.6 | 0.23 ± 0.01 | 1.9 ± 0.2 |
| Xanthine (**1**), pH 8.5[d] | 0.22 ± 0.01 | 2.6 ± 0.3 | 0.16 ± 0.02 | 1.9 ± 0.2 |
| Caffeine (**2**) | 0.51 ± 0.02 | 2.57 ± 0.03 | 0.39 ± 0.02 | 1.9 ± 0.1 |
| Theophylline (**3**), pH 8.0 | 0.55 ± 0.02 | 1.55 ± 0.08 | 0.43 ± 0.01 | 3.01 ± 0.07 |
| Theophylline (**3**), pH 8.5[d] | 0.40 ± 0.01 | 2.22 ± 0.08 | 0.30 ± 0.07 | 2.0 ± 0.6 |
| Paraxanthine (**4**), pH 8.0 | 0.48 ± 0.03 | 2.11 ± 0.08 | 0.44 ± 0.03 | 1.9 ± 0.1 |
| Paraxanthine (**4**), pH 8.5[d] | 0.36 ± 0.04 | 2.88 ± 0.05 | 0.20 ± 0.04 | 2.0 ± 0.1 |
| Theobromine (**5**) | 0.58 ± 0.04 | 1.9 ± 0.6 | 0.42 ± 0.01 | 1.80 ± 0.06 |
| Uric acid (**6**), pH 8.0 | 0.087 ± 0.006 | 2.5 ± 0.2 | 0.060 ± 0.003 | 1.98 ± 0.07 |
| Uric acid (**6**), pH 8.5[d] | 0.113 ± 0.004 | 2.8 ± 0.4 | 0.06 ± 0.01 | 2.0 ± 0.6 |
| 1-Methyluric acid (**7**) | 0.096 ± 0.004 | 2.5 ± 0.1 | 0.101 ± 0.001 | 1.84 ± 0.07 |
| 1,3-Dimethyluric acid (**8**), pH 8.0 | 0.072 ± 0.001 | 2.64 ± 0.05 | 0.088 ± 0.004 | 2.0 ± 0.1 |
| 1,3-Dimethyluric acid (**8**), pH 8.5[d] | 0.099 ± 0.001 | 2.8 ± 0.2 | 0.064 ± 0.007 | 1.9 ± 0.3 |
| 1,7-Dimethyluric acid (**9**), pH 8.0 | 0.119 ± 0.008 | 3.0 ± 0.2 | 0.113 ± 0.008 | 1.7 ± 0.1 |
| 1,7-Dimethyluric acid (**9**), pH 8.5[d] | 0.109 ± 0.001 | 2.6 ± 0.3 | 0.061 ± 0.002 | 1.8 ± 0.1 |
| 3,7-Dimethyluric acid (**10**) | 0.090 ± 0.009 | 3.4 ± 0.4 | 0.063 ± 0.004 | 2.4 ± 0.4 |
| 1,3,7-Trimethyluric acid (**11**) | 0.07 ± 0.01 | 2.8 ± 0.4 | 0.067 ± 0.003 | 2.4 ± 0.3 |
| 1,3,7,9-Tetramethyluric acid (**12**) | 0.121 ± 0.002 | 3.0 ± 0.3 | 0.079 ± 0.006 | 2.7 ± 0.2 |
| Allantoin (**13**) | No inhibition | – | No inhibition[e] | – |
| Adenine (**14**) | 12.9 ± 1.0 | 1.6 ± 0.1 | 15.8 ± 0.5 | 2.1 ± 0.1 |
| Uridine (**15**) | 4.6 ± 0.2 | 2.5 ± 0.4 | 3.1 ± 0.1 | 2.3 ± 0.2 |
| Uracil (**16**) | 4.7 ± 0.9 | 3.1 ± 0.8 | 4.2 ± 0.2 | 3.0 ± 0.5 |
| Uracil-4-acetic acid (**17**) | 3.17 ± 0.03 | 2.4 ± 0.2 | 2.6 ± 0.1 | 2.3 ± 0.3 |

[a] Errors are the standard deviations obtained from triplicate determinations of $IC_{50}$ values.
[b] Concentration of inhibitor required to yield 50% of the activity (i.e., $v_i/v_o = 0.5$) observed when CTPS (26 μg/mL) is assayed with 150 mM $NH_4Cl$.
[c] Concentration of inhibitor required to yield 50% of the activity observed when CTPS (26 μg /mL) is assayed with Gln (10 mM) and GTP (0.15 mM).
[d] Assays at pH 8.5 were conducted in TAPS buffer (70 mM, pH 8.5) rather than Hepes buffer (70 mM, pH 8.0).
[e] Approximately 20% reduction in $v_i/v_o$ was observed at [allantoin] = 37.57 mM.

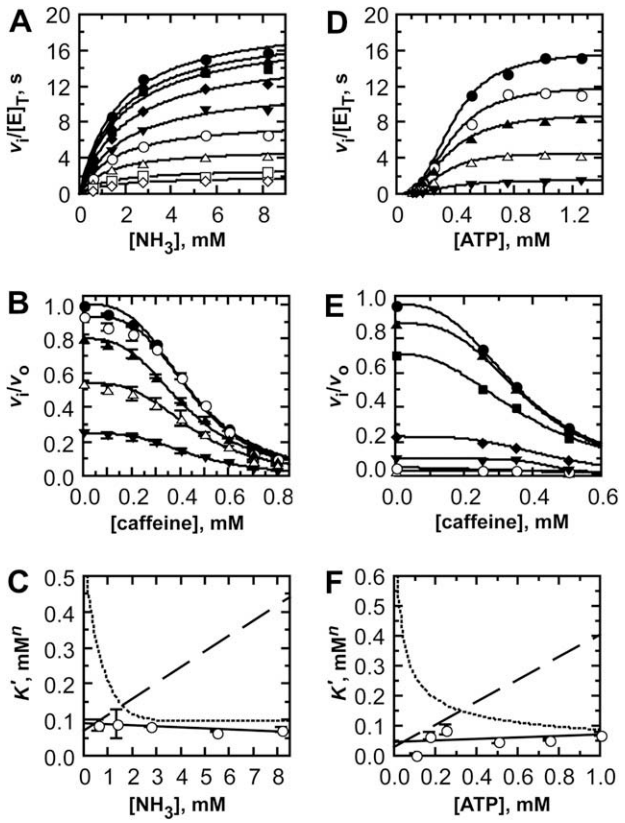



**Figure 2.** Inhibition of CTPS-catalyzed $NH_3$-dependent CTP formation with respect to $NH_3$ (A–C) and ATP (D–F) as substrates. Error bars are the standard deviations. Panel A: The dependence of the initial velocities ($v_i$) on [$NH_3$] in the presence of varying [caffeine] (0 mM, ●; 0.1 mM, ▲; 0.2 mM, ■; 0.3 mM, ◆; 0.4 mM, ▼; 0.5 mM, ○; 0.6 mM, △; 0.7 mM, □; and 0.8 mM, ◇) is shown. The standard assay was conducted in Hepes buffer (70 mM, pH 8.0) containing EGTA (0.5 mM), $MgCl_2$ (10 mM), CTPS (20 μg/mL), $NH_4Cl$ (10, 25, 75, 100, and 150 mM), ATP (1 mM), and UTP (1 mM) in a total volume of 1 mL. Initial velocities were fit to eqn. 1 by nonlinear regression analysis using *KaleidaGraph* v. 4.0 (Synergy Software, Reading, PA). Panel B: The effect of caffeine on the relative initial velocities for $NH_3$-dependent CTP formation ($v_i/v_o$) is shown for various [$NH_3$] (8.16 mM, ●; 5.44 mM, ○; 2.72 mM, ▲; 1.36 mM, △; and 0.544 mM, ▼). Values of $v_i/v_o$ are relative to $v_i/v_o$ obtained with [$NH_3$] = 8.16 mM and the curves shown are for data fit to eqn. 1. Panel C: The dependence of $K'$ on [$NH_3$] is shown. Theoretical curves for competitive (dashed line) and uncompetitive (dotted line) inhibition were calculated using $K_S$ = 1.83 mM (assuming that $K_S \approx K_m$),[3] $K'$ = 0.080 mM, and eqns. 2 and 3, respectively. Panel D: The dependence of $v_i$ on [ATP] in the presence of varying [caffeine] (0 mM, ●; 0.25 mM, ○; 0.35 mM, ▲; 0.5 mM, △; and 0.75 mM, ▼) is shown. Standard assay conditions were employed with [$NH_4Cl$] = 150 mM. Panel E: The effect of caffeine on $v_i/v_o$ (relative to $v_i/v_o$ for [ATP] = 1 mM) for $NH_3$-dependent CTP formation is shown for various [ATP] (1 mM, ●; 0.75 mM, ▲; 0.5 mM, ■; 0.25 mM, ◆; 0.167 mM, ▼; and 0.10 mM, ○). Curves are fits to eqn. 1. Panel F: The dependence of $K'$ on [ATP] is shown. Theoretical curves for competitive (dashed line) and uncompetitive (dotted line) inhibition were calculated using $K_S$ = 0.066 mM (assuming that $K_S \approx K_m$), $K'$ = 0.053 mM, and Eqs. 2 and 3, respectively. In both panels C and F, the linear regression lines (solid lines) show no significant dependence on [substrate], consistent with noncompetitive, multisite inhibition.

**Table 2**
Kinetic parameters for the multisite inhibition of CTPS-catalyzed $NH_3$-dependent CTP formation by caffeine[a]

| [$NH_3$], mM | $K'$ | $IC_{50}$, mM | $n$ |
|---|---|---|---|
| 8.16 | 0.073 ± 0.007 | 0.44 ± 0.01 | 3.2 ± 0.1 |
| 5.44 | 0.067 ± 0.002 | 0.45 ± 0.01 | 3.4 ± 0.1 |
| 2.72 | 0.082 ± 0.002 | 0.42 ± 0.01 | 2.9 ± 0.1 |
| 1.36 | 0.090 ± 0.040 | 0.45 ± 0.04 | 3.1 ± 0.1 |
| 0.54 | 0.086 ± 0.015 | 0.44 ± 0.01 | 3.0 ± 0.2 |
| Average | 0.080 ± 0.017 | 0.44 ± 0.02 | 3.1 ± 0.4 |

[a] Values are means of three experiments; errors are standard deviations.

formation, respectively (Table 1). This observation is consistent with our previous report that 8-oxoguanosine inhibited $NH_3$- and Gln-dependent CTP formation with $IC_{50}$ values that were reduced 2.2- and 4.0-fold, respectively, relative to guanosine.[22]

Xanthine (**1**, $pK_{a3}$ = 7.7, $pK_{a7/9}$ = 10.6), paraxanthine (**4**, $pK_{a3}$ = 8.8), and theophylline (**3**, $pK_{a7/9}$ = 8.7) are partially ionized under the assay conditions.[40] To determine the extent to which ionization affects inhibition, $IC_{50}$ values were also determined at pH 8.5 for these compounds and their corresponding uric acids (**6**, **9**, and **8**, respectively). Raising the pH from 8.0 to 8.5 resulted in slightly increased inhibition of $NH_3$-dependent CTP formation by the xanthines; however, the inhibition was slightly decreased (**6** and **8**) or remained roughly unchanged (**9**) for the corresponding uric acids. On the other hand, raising the pH from 8.0 to 8.5 for Gln-dependent CTP formation caused enhanced inhibition for xanthines **1**, **3**, and **4**, as well as for the methylated uric acids **8** and **9**. Only the $IC_{50}$ value for uric acid was unchanged. The origin of this pH-effect on the inhibition of Gln-dependent CTP formation is unclear although it likely arises due to changes in the ionization state of the enzyme affecting Gln- or GTP-binding since the $IC_{50}$ value of **8** was reduced 1.4-fold when the pH was increased yet there is no significant change in the ionization state of **8** within this pH range ($pK_{a9}$ = 5.75, $pK_{a3}$ = 10.3).[40] Overall, it appears that an increased negative charge on the purine ring yields better inhibition as is evidenced by the lower $IC_{50}$ values observed for the xanthines at pH 8.5 and the observation that the uric acids, which are fully ionized at the 7- or 9-position ($pK_{a7/9} \approx 5.8$–5.9) under our assay conditions, are the most potent inhibitors. To test this conclusion, we examined inhibition by uracil-4-acetic acid (**17**). Indeed, the addition of the negative carboxylate resulted in a 1.5-fold reduction in the $IC_{50}$ value relative to that of uracil (**16**) or uridine (**15**). However, our observation that **17** was a relatively weak inhibitor and that allantoin (**13**) was not an inhibitor indicated that an intact purine ring is required for potent inhibition.

It is possible that inhibition of CTPS by the xanthines and uric acids could arise due to 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.[7,41,42] However, using gel filtration-HPLC as described previously,[22] we have shown that neither caffeine nor 3,7-dimethyluric acid at concentrations of 1 mM (i.e., 2× and 10× their $IC_{50}$ values, respectively) appear to impair the ability of CTPS to form tetramers under the assay conditions (i.e., in the presence of 1 mM each of ATP and UTP). Since the Hill numbers ($n$) for inhibition by most of the compounds range between 2.0 and 4.0, there is cooperativity in inhibitor binding. Such cooperativity likely arises from the interaction between subunits within the intact CTPS tetramer.

At present, the location of the inhibitor binding site is not known. However, the fact that adenosine ($IC_{50}$ = 11 mM for the $NH_3$-dependent reaction[22]), adenine, uridine, and uracil (Table 1) are much weaker inhibitors than the xanthines and uric acids suggests that the purine-based inhibitors are not binding at the ATP- or UTP-binding sites. This conclusion is also supported by our observation that caffeine behaves as a noncompetitive, multisite inhibitor of CTPS with respect to ATP (Fig. 2). Moreover, the purine-based inhibitors do not appear to disrupt the ATP/UTP-dependent formation of CTPS tetramers.

Thus, inhibition of *E. coli* CTPS can be achieved solely through interactions between the enzyme and a xanthine heterocycle, suggesting that the purine ring may serve as a useful scaffold for the development of CTPS inhibitors. Studies to characterize the architecture of the inhibitor binding site are currently underway.

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