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THE SYNTHESIS OF 5-AZIDO-UTP AND UDP-PYRIDOXAL AND THEIR EFFECT ON THE CARBAMOYL-PHOSPHATE SYNTHETASE (CPS II) IN THE MAMMALIAN MULTIENZYME POLYPEPTIDE CAD

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Abstract: 5-azidouridine-5'-triphosphate and UDP-pyridoxal have been synthesized using modified synthetic methods. The two synthetic UTP analogues, with commercially available analogues and chemicals have been tested for their effect on CPSase II in the mammalian multienzyme polypeptide CAD. Structure activity relationships are discussed.

Pyrimidine biosynthesis in mammalian cells is initiated by the three enzyme activities found in the multifunctional polypeptide CAD [reviewed in 1]. The three enzymes are the glutamine-dependent carbamoyl-phosphate synthetase (CPSaseII), aspartate trans-carbamoylase (ATCase) and dihydroorotase (DHOase). CPSaseII is allosterically controlled by UTP (negative effector) and phosphoribosyl pyrophosphate PRibPP (positive effector) [2,6].

Reactive nucleotide analogues have provided valuable information on the structure and mechanism of a number of enzymes. The mechanism by which the interaction of metabolites with the enzyme leads to modulation of its activity are clearly of interest. Furthermore, the biosynthesis of pyrimidines is essential for most growing cells, thus the design and synthesis of analogues for the metabolites are a potential target for the drug discovery for chemotherapy against cancer. We have used two synthetic analogues, other nucleotides and chemicals to compare the structures of effectors and the enzyme activity of the glutamine-dependent carbamoyl-phosphate synthetase (CPSaseII) domain of CAD, to examine the effect of UTP analogues on CPSase II in the multienzyme polypeptide CAD and demonstrate the importance of the structures of effector analogues to the CPSase II activities. The studies will increase our understanding of the binding sites of PRibPP and UTP in the mammalian multienzyme polypeptide CAD, which regulate the biosynthesis of pyrimidines [3].

The synthetic routes to UDP-pyridoxal are outlined in Scheme 1. UDP-pyridoxal **3** was synthesized by a modification of previously published methods [4a, 4b] and from the coupling of pyridoxal 5-phosphate **1** and UMP-morpholidate **2** in anhydrous pyridine. The extracted aqueous layer was applied to an anion exchange column of Dowex 1-X8 (Cl^- form) to remove UMP and pyridoxal 5-phosphate, the yield was 65%. 5-azidouridine 5'-triphosphate

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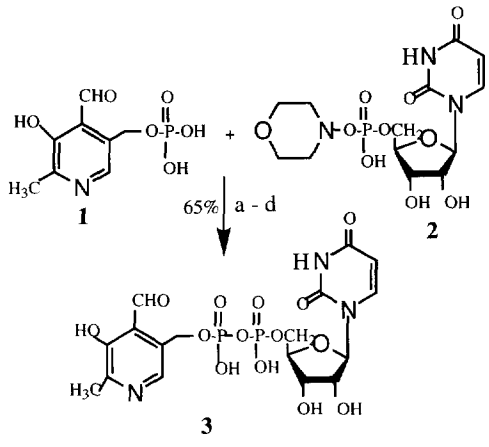
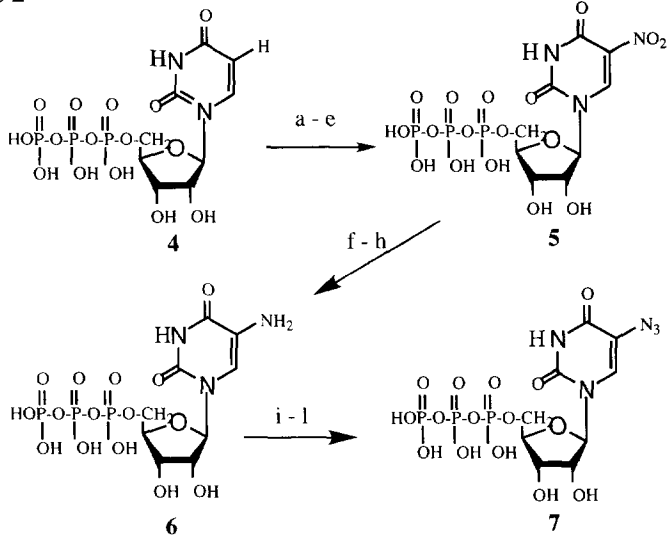
Scheme 1



Reagents and conditions: a) Pyridine (anhydrous), 3-5 days, rt; b) Extracted with ethylether; c) Dowex 1-X8 (Cl^- form), washed with H_2O, 3mM-10mM LiCl, eluted with 10mM HCl; d) Adjusted pH to 6.5 with 1N LiOH, kept 0°C, 1h, centrifuged, dried.

Scheme 2



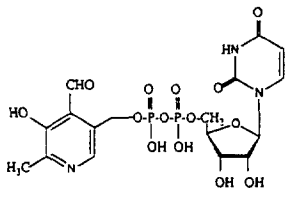
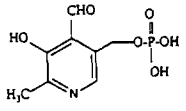
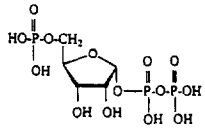
Reagents and conditions: a) Dowex 50W resin column (H^+ form); b) $NOBF_4$/DMF (anhydrous); c) 1N HCl, 15 min, neutralized by NH_4OH; d) Sephadex G-10 column (H_2O); e) Benzyl diethylaminoethyl cellulose column (gradient of NH_4HCO_3); f) Zn/HCl, 0.5-2h, neutralized by NH_4OH; g) Diethylaminoethyl cellulose column (gradient of NH_4HCO_3); h) Sephadex G-10 column (H_2O); i) NaN_3, 10 min, 0°C, 30 min, rt; j) Sephadex G-10 column (H_2O); k) Benzyl diethylaminoethyl cellulose column (gradient of NH_4HCO_3); l) Dowex 50W column (H_2O). Overall yield:37.5% [5].

(N_3-UTP) **7** was chemically synthesized by modification of a previously published synthetic method for 5-azido-2'-deoxyuridine 5'-mono-phosphate [4c] and the other chemical synthetic methods for nucleotides [4d-e]. The synthetic routes to N_3-UTP **7** are outlined in Scheme 2. UTP **4** (sodium salt) was firstly converted to the free acid form by applying the nucleotide to a Dowex 50W resin (H^+ form) column. The dried UTP was nitrated by nitrosonium tetrafluoroborate. The neutralized sample was desalted using Sephadex G-10 column and then an anion and cation mixed bed exchange benzyl diethylaminoethyl cellulose column to remove mixed ions. 5-NO_2UTP **5** was reduced to 5-NH_2UTP **6** by adding the mixture of clean granulated zinc and HCl. Then the neutralized 5-NH_2UTP **6** was applied to an anion exchange diethylaminoethyl cellulose column and desalted by Sephadex G-10 gel filtration chromatography. 5-NH_2UTP **6** was finally converted to the product N_3-UTP **7** by the addition of NaN_3 which was produced from the mixture of $NaNO_2$ and H_2O. The neutralized sample was again desalted by gel filtration chromatography on a Sephadex G-10 column, a mixed bed benzyl diethylaminoethyl cellulose column and a strongly acidic cation Dowex 50W column to remove residual NH_4^+. The overall yield was 37.5% from UTP.

The two synthetic UTP analogues UDP-pyridoxal **3** and N_3-UTP **7** , with commercially available analogues and chemicals: uridine 5'-diphosphate (UDP) **8,** uridine (Urd) **9**, pyridoxal 5-phosphate **12**, pyridoxal hydrochloride **13**, D-Ribose-5-phosphate **11** and 1'-phospho-ribosyl-5'-pyrophosphate (PRibPP) **10** have been tested for their effect on CPSase II in CAD. Table I shows the results that 5-azidouridine-5'-triphosphate **7**, uridine 5'-diphosphate **8** and pyridoxal hydrochloride **13**, like UTP **1**, inhibit the activity of the glutamine-dependent carbamyl phosphate synthetase (CPSase II) in the mammalian multienzyme polypeptide CAD. However, UDP-pyridoxal **3**, pyridoxal 5-phosphate **12**, like PRibPP **10**, activate CPSase II, while uridine **9** and D-ribose 5-phosphate **11** have no effect. Tamura et al. have demonstrated that ADP-pyridoxal is a potent inhibitor of yeast alcohol dehydrogenase and rabbit muscle pyruvate kinase [4b], which promted our interest to use UDP-pyridoxal as an UTP analogue to inhibit the activity of CPS II. However, the results show that UDP-pyridoxal, not as expected as an inhibitor, activates the enzyme. Comparing the structures of UTP **1** and UDP-pyridoxal **3**, the major difference between two compounds is that pyridoxal group in the UDP-pyridoxal replaces the γ-position phosphate in UTP, to cause the totally opposite effect on the CPSase II activities. Obviously, the structure of this part of the effectors is really important to the activity of the enzyme. Table I demonstrates that UDP **8** is also a good inhibitor of the activity of the enzyme even though lacking one phosphate group compared to UTP **1**. However, very interestingly, Uridine **9** has completely no effect, which suggests that phosphate groups in UTP are the most important groups to inhibit the activity on CPSase II in the mammalian multienzyme polypeptide CAD. On the other hand, where the 5-H in UTP **1** has been replaced by an azido group, the UTP analogue N_3-UTP **7**, inhibits the activity of CPSase II, but is much less efficiency than UTP. Table I shows that 52% activity of the CPSase II has been inhibited by the same concentration of N_3-UTP **7** and 82% by UTP **1**. The results suggest that the 5-H position or the base in UTP is one of the

Table I. Effect of nucleotides and chemicals on the activity of CPSase II in the multienzyme polypeptide CAD

No. of Entry	Nucleotides and chemicals	Activity	Concentration
		%	mM
1	ATP (Substrate only)	[100]	[4]
2	+ **1**	18	1.2
3	+ **7**	48	1.2
4	+ **8**	54	1.2
5	+ **13**	73	1.2
6	+ **9**	100	1.6
7	+ **11**	100	1.6

8	+3		120	0.4
9	+12		170	0.4
10	+10		320	0.4

The activities were determined using the method given in [7]. pH for all used analogues and chemicals were adjusted to 7.5 before use.

most important positions to cause the inhibition of the enzyme activity. Thus, a single change in structure at the both 5-H position and phosphate position of UTP has a dramatic effect, changing the enzyme activity.

We believe that the reason that UDP-pyridoxal **3** activates the enzyme is the presence of pyridoxal 5-phosphate **12**. Table I shows that pyridoxal 5-phosphate has increased the activity of the CPSase II by 70% and UDP-pyridoxal by only 20%. The results suggest that the partial structure of UDP-pyridoxal **3**, the pyridoxal 5-phosphate **12**, is the cause for the activation of the enzyme activity. Pyridoxal hydrochloride **13** has been used to investigate if the pyridoxal group activates the enzyme activity, and the result demonstrates that pyridoxal itself, without one more crucial phosphate group, inhibits the activity of the enzyme (27% inhibition). The evidences suggest, once again, that the phosphate group of UTP is the most important group to act as an efficient allosteric inhibitor. The phosphate group has a crucial role not just for the allosteric inhibitor UTP, but also for the allosteric activator PRibPP, to regulate the biosynthesis of pyrimidine. The experimental results demonstrate that the analogue D-Ribose-5-phosphate **11**, unlike PRibPP **10**, has no effect on the CPSase II. Comparing the structures of both PRibPP **10** and D-Ribose-5-phosphate **11**, it is not difficult to draw a conclusion that D-Ribose-5-phosphate does not inhibit or activate the enzyme because D-Ribose-5-phosphate has two missing phosphate groups, of which one is 1'-phosphate and another is 5'-phosphate in PRibPP.

The two synthetic analogues UDP-pyridoxal **3** and N_3-UTP **7**, with commercially available analogues and chemicals used on the activity of CPSase demonstrate how important the structure of effectors is to the activity of the enzyme. The results will certainly help the design of more efficient analogues for the metabolites which regulate the biosynthesis of pyrimidine and the drug discovery for chemotherapy against cancer.

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5. Abbreviations: CAD, the multienzyme polypeptide carrying the activities carbamoyl phosphate synthetase II aspartate transcarbamoylase and dihydroorotase; BD-Cellulose, benzyl diethylaminoethyl-Cellulose; DEAE-Cellulose, diethylaminoethyl-Cellulose; 5-N_3UTP, 5-Azidouridine-5'-triphosphate; PRibPP, 1'-phospho-ribosyl-5'-pyrophosphate.
6. Enzymes: aspartate transcarbamoylase (EC 2.1.3.2., ATCase); glutamine-dependent carbamoyl-phosphate synthase (EC 6.3.4.16., CPSase II); dihydroorotase (EC 3.5.2.3. DHOase). the baby hamster kidney cell line 165-28 was a gift from Professor D.R. Evans, Wayne State University, Detroit.
7. Assay procedure: Carbamoyl-phosphate synthase was assayed by a modification of previously methods [2]. A total incubation volume of 250µl contained 0.1M Tris, pH 7.5 at 37°C, 0.1M KCl, 7.5% dimethyl sulphoxide (DMSO), 2.5% glycerol, 5 mM $MgCl_2$, 4 mM ATP, 1 mM dithiothreitol, and nitrogen donor either glutamine (3 mM) or NH_4Cl, pH 7.5 (40 mM). The reaction was initiated by adding 10µl of CAD solution (1-3µg of protein) and 15 µl of $NaH^{14}CO_3$ (0.2M, 0.75Ci/mol). After 15 min, the assay was stopped by mixing with 0.10 ml of 2 M NH_4Cl and heating the tubes at 90°C for 20 min. The unchanged $NaHCO_3$ was driven off as CO_2 by adding 0.1 ml of 14% (v/v) $HClO_4$, and the assay mixtures were dried and resuspended for scintillation counting.

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