Synthesis and Properties of the Naturally Occurring *N*-[(9-β-D-Ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]-L-threonine (mt^{6}A) and Other Related Synthetic Analogs†

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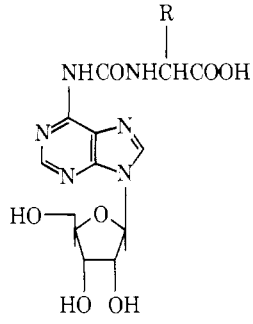
ABSTRACT: The naturally occurring modified nucleoside, *N*-[(9-β-D-ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]-L-threonine (mt^{6}A), and the corresponding glycine analog mg^{6}A were synthesized from N^6-methyl-2′,3′,5′-tri-*O*-acetyladenosine and the appropriately blocked isocyanates derived from threonine and glycine. The natural mt^{6}A isolated from *Escherichia coli* tRNA (F. Kimura-Harada et al. (1972), *Biochemistry 11,* 3910), from wheat embryo tRNA (R. Cunningham and M. W. Gray (1974), *Biochemistry 13,* 543), and from rat liver tRNA (Rogg et al. (1975), *Eur. J. Biochem. 53,* 115) was found to be identical with the synthetic mt^{6}A in paper and thin-layer chromatography and electrophoresis. Several analogs of the parent 6-ureidopurine ribonucleoside, *N*-[(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]-L-thronine (t^{6}A), were also prepared. Starting from 2′,3′,5′-tri-*O*-acetylguanosine and 2′,3′,5′-tri-*O*-acetylcytidine and the above isocyanates, the t^{6}A analogs, *N*-[(9-β-D-ribofuranosyl-6-oxo-1*H*-purin-2-yl)carbamoyl]-L-threonine (t^{2}G) and *N*-[(1-β-D-ribofuranosyl-2-oxopyrimidin-4-yl)carbamoyl]-L-threonine (t^{4}C), were prepared. Also synthesized were the corresponding glycine analogs, g^{2}G and g^{4}C, from guanosine and cytidine, respectively. The 2′-deoxyribosyl analog, *N*-[(9-β-D-2′-deoxyribofuranosylpurin-6-yl)carbamoyl]-L-threonine (2′-deoxy-t^{6}A), and the arabinosyl derivative, *N*-[(9-β-D-arabinofuranosylpurin-6-yl)carbamoyl]-L-threonine (t^{6}AraA), were synthesized from the appropriate urethane and the requisite amino acid. The ureido group in mt^{6}A could not be hydrolyzed by the enzymes urease, peptidase, and protease. Various chemical and biological properties of the naturally occurring mt^{6}A and the related analogs are discussed.

The anticodon adjacent hypermodified nucleoside, *N*-[(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]-L-threonine (t^{6}A, **1**),[1] has been isolated and characterized from tRNA



1, t^{6}A, R = CH(OH)CH$_3$
2, g^{6}A, R = H

of many organisms (Chheda et al., 1969; Schweizer et al., 1969). It has also been isolated and characterized from human and rat urine (Chheda, 1969). A number of papers concerning its specific location in tRNA species have appeared (Takemura et al., 1969; Ishikura et al., 1969; Harada and Nishimura, 1972). Studies related to biosynthesis of t^{6}A and its significance in biological systems have recently been reported (Chheda et al., 1972; Powers and Peterkofsky, 1972; Korner and Soll, 1974, and Elkins and Keller, 1974). A glycine analog of t^{6}A, *N*-[(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]glycine (g^{6}A, **2**), has also been isolated from yeast tRNA (Schweizer et al., 1970). There have been suggestions that several other modified nucleosides with the general structure of 6-ureidopurines are present in *Escherichia coli* tRNA (Hall, 1964; Ishikura et al., 1969; Cory and Marcker, 1970; Harada et al., 1971; Harada and Nishimura, 1972). Nishimura and his associates (Kimura-Harada et al., 1972) have isolated and characterized *N*-[(9-β-D-ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]-L-threonine (mt^{6}A) from *E. coli* tRNA^{Thr}. More recently Cunningham and Gray (1974) and Rogg et al. (1975) also isolated this nucleoside from wheat embryo tRNA and rat liver tRNA, respectively.

This paper describes the synthesis and chemical and biological properties of mt^{6}A (**5**) and its glycine analog, mg^{6}A (**6**). Described herein are also the syntheses and properties of the carbamoylthreonine and carbamoylglycine derivatives of 2′-deoxyadenosine, guanosine, cytidine, and 9-(β-D-arabinofuranosyl)adenine.

Experimental Section

Melting points were determined on Mel-Temp laboratory model melting point apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian A-60A and XL-100 spectrometer with $(CH_3)_4Si$ as an internal reference unless stated otherwise. Uv spectra

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[1] Abbreviations used are: t^{6}A, *N*-(purin-6-ylcarbamoyl)-L-threonine ribonucleoside; g^{6}A, *N*-(purin-6-ylcarbamoyl)glycine ribonucleoside; mt^{6}A, *N*-[(9-β-D-ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]-L-threonine; mg^{6}A, *N*-[(9-β-D-ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]glycine; 2′-deoxy-t^{6}A, *N*-[(9-β-D-2′-deoxyribofuranosylpurin-6-yl)carbamoyl]-L-threonine; 2′-deoxy-g^{6}A, *N*-[(9-β-D-2′-deoxyribofuranosylpurin-6-yl)carbamoyl]glycine; t^{6}AraA, *N*-[(9-β-D-arabinofuranosylpurin-6-yl)carbamoyl]-L-threonine; t^{2}G, *N*-[(9-β-D-ribofuranosyl-1*H*-6-oxopurin-2-yl)carbamoyl]-L-threonine; g^{2}G, *N*-[(9-β-D-ribofuranosyl-1*H*-6-oxopurin-2-yl)carbamoyl]glycine; t^{4}C, *N*-[(1-β-D-ribofuranosyl-2-oxopyrimidin-4-yl)carbamoyl]-L-threonine; g^{4}C, *N*-[(1-β-D-ribofuranosyl-2-oxopyrimidin-4-yl)carbamoyl]glycine.

Table I: Ultraviolet Spectral Maxima and Paper Chromatographic Mobilities of the mt^{6}A and Related Compounds.

Compound	Uv max (nm) ($\epsilon \times 10^{-3}$)			Paper Chromatography $R_f \times 100$ in Solvents	
	0.1 *N* HCl	H_2O	0.1 *N* NaOH	A	B
1, t^{6}A	272 (20.4) 277 (21.6)	270 (22.9) 277 (19.4)	270 (16.3) 277 (16.0) 299 (11.1)	31	47
2, g^{6}A	271 (18.2) 276 (19.1)	269 (20.9) 276 (17.1)	270 (13.5) 277 (13.8) 298 (12.8)	20	20
3a	261 (17.7)	264 (16.6)	264 (16.9)	77	92
4a	275 (17.7) 283 (16.5)	275 (17.2) 283 (16.1)	278 (16.5) 284 (15.7)		
5, mt^{6}A	277 (22.3) 283 (22.3)	278 (23.5) 284 (23.1)	277 (22.5) 283 (21.5)	44	46
6, mg^{6}A	278 (sh)[a] 283 (16.7)	277 (17.1) 284 (16.7)	277 (15.4) 284 (15.8)	43	26
7	277 (19.8) 284 (19.8)	277 (20.4) 284 (19.8)	277 (19.6) 284 (18.9)	31	61
10	270 (16.1)	265 (17.3)	291 (25.4)	86	90
11	265 (16.3) 271 (15.4)	265 (17.1)	289 (25.2)	84	93
12, 2′-deoxy-t^{6}A	275 (21.3) 270 sh (19.4)	269 (21.2) 275 (18.2)	269 (14.9) 277 (15.3) 294 (11.4)	33	44
13, 2′-deoxy-g^{6}A	275 (22.4) 270 sh (20.0)	268 (22.6) 274 (19.2)	269 (14.9) 277 (15.3) 294 (11.4)	31	43
14, t^{6}AraA	275 (22.2) 269 sh (20.0)	269 (22.7) 275 (19.3)	270 (18.6) 276 (17.5) 294 (9.8)	39	34
16	258 (16.4)	258 (14.3)	260 (14.1)		
18, t^{2}G	258 (19.0)	256 (19.8) 270 (sh)	265 (16.8)	15	21
19, g^{2}G	260 (20.0)	257 (21.1) 270 (sh)	266 (19.0)	11	18
20	276 (13.4)	269 (9.6)	270 (9.4)		
23, t^{4}C	293 (14.8) 240 (11.4)	286 (14.5) 242 (11.9)	292 (20.7)	34	27
24, g^{4}C	296 (13.8) 241 (sh)	286 (12.0) 242 (sh)	292 (19.8)	30	24

[a] sh, shoulder.

were recorded on a Cary 14 or Beckman Acta-V spectrophotometer and the ir spectra were recorded in KBr pellet on a Perkin-Elmer 457 or Infracord spectrophotometer. Optical rotations were determined on a Perkin-Elmer Model 141 polarimeter using a 10-cm, 1-ml micro cell. Thin-layer chromatography (TLC) was carried out on glass plates coated with silica gel PF-254 and paper chromatography was carried out on Whatman No. 1 paper in the following solvent systems: (A) *i*-PrOH–H_2O–concentrated NH_4OH (7:2:1), (B) EtOAc–2-ethoxyethanol–16% HCOOH (4:1:2, upper phase), (C) EtOAc–*n*-PrOH–H_2O (4:1:2, upper phase), (D) *n*-PrOH–concentrated NH_4OH–H_2O (55:10:35), and (E) EtOAc–EtOH (49:1). Preparative TLC was performed on glass plates coated with silica gel PF-254 (E. Merck AG). Elemental analyses were performed by Heterocyclic Chemical Corp., Harrisonville, Mo.

2′,3′,5′-Tri-O-acetyl-N^6-methyladenosine (**3a**). A suspension of N^6-methyladenosine (1.40 g; 5.0 mmol) in 5 ml of anhydrous pyridine was allowed to react with 5 ml of acetic anhydride at room temperature for 2 hr. To the clear solution, 5 ml of methanol was added and the mixture was evaporated to dryness in vacuo (ca. 45°). Last traces of pyridine were removed by repeated evaporation with toluene to yield 1.83 g (85.9%) of a gummy residue. This crude material was found to be homogeneous by TLC in solvents B, C, and E. The uv spectra of the product were similar to N^6-methyladenosine (Table I). This material was used without further purification for the next step. For analysis 100 mg of the product was passed through a silica gel column (100–200 mesh; 2.2 × 24 cm) and the column was eluted with 250 ml of $CHCl_3$–MeOH (19:1). The fractions between 125 and 175 ml containing the desired product were evaporated to dryness to give a gummy residue. This material was further dried under high vacuum at 61° for 20 hr. Anal. Calcd for $C_{17}H_{21}N_5O_7 \cdot H_2O$: C, 48.00; H, 5.45; N, 16.46. Found: C, 47.75; H, 5.44; N, 16.22.

*N-[9-(2′,3′,5′-Tri-O-acetyl-β-*D*-ribofuranosylpurin-6-yl)-N-methylcarbamoyl]-O-benzyl-*L*-threonine Benzyl Ester* (**4a**). To a solution of 1.06 g (2.5 mmol) of **3a** in 10 ml of anhydrous pyridine was added 1.63 g (5.0 mmol) of the isocyanate of *O*-benzyl-L-threonine benzyl ester (Chheda and Hong, 1971). The mixture was heated with stirring in a glass bomb at 90° for 6 hr. The reaction mixture was then evaporated to dryness and the last traces of pyridine repeatedly azeotroped with toluene. The residue (by TLC) showed that it contained the starting material, the desired product (determined by uv on TLC band), and asmall amount of some fast moving material. The reaction product was purified by column chromatography over silica gel (100–200 mesh; 3.5 × 90 cm). The column was eluted

first with $CHCl_3$ (500 ml) and then with $CHCl_3$–MeOH (19:1, 1000 ml). The desired product was obtained in these later fractions. The fractions were combined and evaporated to dryness to give 1.33 g (70.0%) of the pure product **4a** as a gummy residue, which exhibited a single spot in TLC in solvents B and E. Anal. Calcd for $C_{36}H_{40}N_6O_{11}\cdot 1.5H_2O$: C, 56.91; H, 5.70; N, 11.06. Found: C, 56.49; H, 5.62; N, 10.84.

N-[(*9-β-*D-*Ribofuranosylpurin-6-yl*)-*N-methylcarbamoyl*]-L-*threonine* (*mt⁶A*, **5**). A solution of 1.20 g of the above benzyl ester (**4a**) in 50 ml of MeOH was hydrogenated under atmospheric pressure and at room temperature with 400 mg of PdO. The hydrogenation was continued till there was no further consumption of H_2 (16 hr). The catalyst was filtered, and the solvent was removed under reduced pressure. TLC of the residue in solvents B and C showed only one spot different from the starting material. The syrupy residue was dissolved in 40 ml of 4 *N* NH_3–MeOH and the mixture was stirred at 4° for 4 hr. The solution was then evaporated to dryness and the residue was dissolved in 10 ml of absolute EtOH, and cooled at −20°. The crystalline mt⁶A product (**5**) was collected on a filter. The material was recrystallized twice from absolute EtOH: yield 558 mg (76.4%); mp 159–160°; $[\alpha]^{23}D$ −5.31° (*c*, 0.49, H_2O); ir max (cm^{-1}) 1675 (ureido C=O), 1580, and 1520 (C=C, C=N); NMR (data obtained on Varian XL-100) δ (D_2O, external Me_4Si) 1.13 (d, 3, *J* = 6 Hz, $-CHCH_3$), 3.58 (s, 3, $-NCH_3$), 3.78–4.60 (m, 7, 3′-H, α and β -CH, 2′-H, 4′-H, 5′-H), 5.99 (d, 1, *J* = 6 Hz, 1′-H), 8.37 (s, 1,-H), and 8.47 ppm (s, 1,8-H) ($\Delta\delta_{H_8-H_2}$ = 10 Hz); NMR δ (Me_2SO-d_6) 1.03 (d, 3, *J* = 6 Hz, $-CHCH_3$), 3.82 (s, 3, $-NCH_3$), 3.40–4.60 (m, 7, 3′-H, α and β-CH, 2′-H, 4′-H, 5′-H), 6.03 (d, 1, *J* = 6 Hz, 1′-H), 8.54 (s, 1, 2-H), 8.70 (s, 1, 8-H), and 10.41 ppm (d, 1, *J* = 6 Hz, $-NHCH-$) ($\Delta\delta_{H_8-H_2}$ = 16 Hz). Anal. Calcd for $C_{16}H_{22}N_6O_8\cdot 2H_2O$: C, 41.52; H, 5.92; N, 18.16. Found: C, 41.61; H, 5.56; N, 18.09.

Alkaline Hydrolysis of mt⁶A. To a solution of 1.5 mg of mt⁶A (**5**) in 1 ml of water was added 1 ml of 2 *N* NaOH and the mixture was heated in boiling water for 30 min. The solution was then carefully neutralized with 0.1 *N* HCl and examined by TLC and paper chromatography and uv spectra. N^6-Methyladenosine and L-threonine were detected in the hydrolysate. N^6-Methyladenosine was formed in 86.5% yield as quantitated by the uv spectrophotometry of the neutralized hydrolysate.

N-[(*9-β-*D-*Ribofuranosylpurin-6-yl*)-*N-methylcarbamoyl*]*glycine, mg⁶A* (**6**). To a solution of 1.06 g (2.5 mmol) of **3a** in 10 ml of dry pyridine was added 955 mg (5.0 mmol) of the isocyanate of glycine benzyl ester (Hong et al., 1973a). The mixture was heated with stirring in a glass bomb at 90° for 6 hr. TLC of the reaction mixture showed unchanged **3a,** the desired product **4b,** and some fast moving material. The reaction mixture was evaporated to dryness and the residue was fractionated on a silica gel column (100–200 mesh, 2.5 × 65 cm). The column was eluted first with 500 ml of $CHCl_3$ and then with 800 ml of $CHCl_3$–MeOH (49:1). The product **4b** was obtained in these later fractions. The purity of the product was checked by TLC in solvents B, C, and E and by the uv spectra, which were similar to mt⁶A (**5**). The protected product **4b** was debenzylated by hydrogenation over PdO and deacetylated by 4 *N* NH_3–MeOH by the procedures similar to those described for **5.** The final product **6** was crystallized twice from absolute EtOH; overall yield 342 mg (32.7%), mp 173–174°; $[\alpha]^{23}D$ −33.09° (*c*, 0.55, H_2O); ir max (cm^{-1}) 1680 (ureido C=O), 1580 and 1520 (C=C, C=N); NMR δ (D_2O, external Me_4Si) 3.63 (s, 3, CH_3N-), 3.97 (s, 2 $-NH-CH_2$), 3.70–4.50 (m, 5, 3′-H, 2′-H, 4′-H, 5′-H), 6.05 (d, 1, *J* = 6 Hz, 1′-H), 8.50 (s, 1, 2-H), and 8.60 ppm (s, 1, 8-H) ($\Delta\delta_{H_8-H_2}$ = 6 Hz). Anal. Calcd for $C_{14}H_{18}N_6O_7\cdot 2H_2O$; C, 40.16; H, 5.26; N, 20.08. Found: C, 40.30; H, 5.19; N, 20.41.

N-[(*9-β-*D-*Ribofuranosylpurin-6-yl*)-*N-methylcarbamoyl*]*methylamine* (**7**). To a solution of 1.06 g (2.5 mmol) of compound **3a** in 10 ml of anhydrous pyridine was added 0.57 g (10.0 mmol) of methyl isocyanate. The mixture was heated with stirring in a glass bomb at 80° for 6 hr. The brown reaction mixture was then evaporated to dryness in vacuo and the residual pyridine was azeotroped with toluene. The gummy residue was dissolved in 30 ml of 4 *N* NH_3–MeOH and the solution was stirred at 4° for 4 hr. At the end of this period, the precipitated white crystalline material was filtered and washed with MeOH. (The filtrate contained mostly N^6-methyladenosine and some fast moving material as shown by TLC in solvents B and C). The product (**7**) was recrystallized twice from absolute EtOH; yield 620 mg (69.7%), mp 185–186°; ir max (cm^{-1}) 1690 (ureido C=O), 1590 and 1520 (C=C, C=N); NMR δ (Me_2SO-d_6) 3.25 (d, 3, *J* = 10 Hz, $-NHCH_3$), 3.85 (s, 3, $-NCH_3$), 3.70–4.70 (m, 5, 2′-H, 3′-H, 4′-H, 5′-H), 6.10 (d, 1, *J* = 6 Hz, 1′-H), 8.75 (s, 1, 2-H), 8.85 (s, 1, 8-H), and 9.83 ppm (m, 1, $-NH$) ($\Delta\delta_{H_8-H_2}$ = 6 Hz). Anal. Calcd for $C_{13}H_{18}N_6O_5\cdot H_2O$: C, 43.78; H, 5.61; N, 23.57. Found: C, 43.80; H, 5.52; N, 23.65.

Stability Studies on mt⁶A (**5**) *and mg⁶A* (**6**). Compound mt⁶A (**5**) (0.5 mg) and mg⁶A (**6**) (0.5 mg) were dissolved separately in 0.2 ml of each of the following solvents: 1, water; 2, methanol; 3, 0.1 *N* NaOH solution, and then allowed to stand at room temperature. Each solution was examined by TLC in solvents A and B after 1, 2, 4, and 20 hr. The mt⁶A did not show any change in aqueous and methanolic solution, but underwent some degradation to N^6-methyladenosine in the alkaline solution within 1 hr. The mg⁶A did not show any change in water, methanol, and 0.1 *N* NaOH solution at room temperature up to 20 hr.

*Ethyl 9-(3′,5′-Di-O-acetyl-β-*D-*2′-deoxyribofuranosyl)purine-6-carbamate* (**10**). To a suspension of 10.04 g (40 mmol) of 2′-deoxyadenosine in 150 ml of anhydrous pyridine was added 75 ml of acetic anhydride. The reaction mixture was stirred for 40 min and then evaporated to dryness in vacuo.[2] The resulting 2′-deoxy-3′,5′-di-*O*-acetyladenosine (Hayes et al., 1955) was dissolved in 20 ml of pyridine and then 13 ml (ca. 120 mmol) of ethyl chloroformate was added at −10° over a period of 15 min. The reaction mixture was slowly brought to room temperature over a period of 30 min. The suspension was then stirred at room temperature overnight and evaporated to dryness. Traces of pyridine were removed by azeotroping twice with toluene (10 ml each). The residue was dissolved in $CHCl_3$ and the solution was mixed with 100 g of silica gel (100–200 mesh) and the mixture was dried at room temperature. The silica gel was packed on the top of a silica gel column (dry packed, 5.0 × 90 cm), and the column was eluted first with EtOAc (2000 ml) and then with EtOAc–EtOH (49:1). The fractions of 2000–10,000 ml of EtOH–EtOAc (1:49) were

[2] Under these conditions the amino group of adenine does not get acylated. Longer reaction period, however, led to acylation of the amino group and gave the N^6,3′,5′-triacetyl-2′-deoxyadenosine.

pooled and evaporated to dryness. The gummy product weighed 6.99 g (42.0%); ir max (cm^{-1}) 1740 (ester and urethane C=O). Anal. Calcd for $C_{17}H_{21}N_5O_7 \cdot 0.5H_2O$: C, 49.03; H, 5.32; N, 16.82. Found: C, 48.87; H, 5.31; N, 16.47.

N-[(*9-β*-D-*2′-Deoxyribofuranosylpurin-6-yl*)*carbamoyl*]-L-*threonine* (*2′-deoxy-t*^{6}*A*, **12**). A stirred mixture of 1.64 g (3.94 mmol) of the urethane **10** and 0.935 g (7.88 mmol) of L-threonine in 50 ml of anhydrous pyridine was heated in a glass bomb at 120° for 6 hr. The solution was evaporated and the residue was triturated with 10 ml of toluene and reevaporated to dryness. The syrupy residue was then stirred in 100 ml of 4 *N* NH_3–MeOH at room temperature for 5 hr. After evaporating the solvent, the crude product was dissolved in 50 ml of hot EtOH and the solution was diluted with 200 ml of petroleum ether (30–60°). The white solid that separated was collected on a filter and washed with petroleum ether and then with $CHCl_3$, 1.39 g (89.4%). The product, which contained a small amount of 2′-deoxyadenosine and coloring matter, was decolorized in H_2O with charcoal and purified as described below. The aqueous solution was absorbed on the top of a silica gel column (100–200 mesh, 5.0 × 25 cm, dry packed) and the column was allowed to dry. The column was then eluted with solvent C (2000 ml), which removed all the impurities. The column-bound product was eluted with 400 ml of 2.0 *N* NH_4OH in 50% EtOH. After evaporating the solvent to dryness, the residue was dissolved in H_2O and lyophilized. The product (2′-deoxy-t^{6}A, **12**) was then triturated with hot CH_3CN, overall yield 580 mg (37.2%); mp 204–205° effervescent; ir max (cm^{-1}) 1680 (ureido C=O), 1600, and 1525 (C=C, C=N). Anal. Calcd for $C_{15}H_{20}N_6O_7$: C, 45.45; H, 5.08; N, 21.20. Found: C, 45.41; H, 5.25; N, 21.19.

N-[(*9-β*-D-*2′-Deoxyribofuranosylpurin-6-yl*)*carbamoyl*]*glycine* (*2′-deoxy-g*^{6}*A*, **13**). This compound was prepared from the urethane **10** and glycine using the procedures similar to that described for the synthesis of 2′-deoxy-t^{6}A (**12**). The product was crystallized from hot EtOH. The overall yield starting from the urethane **10** was 31.4%. The analytical sample was recrystallized from Me_2SO–EtOH (1:50): mp 172–174° dec; ir max (cm^{-1}) 1680 (ureido C=O), 1620, 1580, and 1525 (C=C, C=N). Anal. Calcd for $C_{13}H_{16}N_6O_6$: C, 44.31; H, 4.57; N, 23.85. Found: C, 44.23; H, 4.58; N, 23.71.

Ethyl 9-(*2′,3′,5′-Tri-O-acetyl-β*-D-*arabinofuranosyl*)*purine-6-carbamate* (**11**). Arabinosyladenine (AraA) when treated with acetic anhydride and pyridine for 1 hr at room temperature gave 2′,3′,5′-tri-*O*-acetyl-9-β-D-arabinofuranosyladenine (**9**) in good yield. A solution of compound **9** in pyridine on treatment with ethyl chloroformate under the conditions similar to those described for the synthesis of **10** gave the desired carbamate **11** in 31.2% yield; ir max (cm^{-1}) 1740 (ester and urethane C=O). Anal. Calcd for $C_{19}H_{23}N_5O_9$: C, 49.03; H, 4.98; N, 15.04. Found: C, 49.02; H, 5.00; N, 14.92.

N-[(*9-β*-D-*Arabinofuranosylpurin-6-yl*)*carbamoyl*]-L-*threonine* (*t*^{6}*AraA*, **14**). A stirred mixture of 698 mg (1.5 mmol) of the urethane **11** and 357 mg (3.0 mmol) of L-threonine in 50 ml of anhydrous pyridine was heated with stirring in a bomb at 120° for 6 hr. At the end of this period, the mixture was allowed to cool to room temperature and the excess threonine was removed by filtration. The filtrate was evaporated to dryness and the residue was azeotroped with 10 ml of toluene. The residue was then stirred in 100 ml of 4 *N* NH_3–MeOH at room temperature for 8 hr and the solvent was evaporated to dryness. The crude product was triturated with 50 ml of EtOH, yield 322 mg (52.1%), mp 183–184° effervescent. The analytical sample was prepared by triturating the product twice with hot EtOH: mp 184–185° effervescent; ir max (cm^{-1}) 1675 (ureido C=O), 1620, 1600, and 1530 (C=C, C=N). Anal. Calcd for $C_{15}H_{20}N_6O_8$: C, 43.69; H, 4.88; N, 20.38. Found: C, 43.65; H, 4.99; N, 20.82.

N-[*9-*(*2′,3′,5′-Tri-O-acetyl-β*-D-*ribofuranosyl*)*-1H-6-oxopurin-2-ylcarbamoyl*]*-O-benzyl*-L-*threonine Benzyl Ester* (**16**). To a solution of 1.02 g (2.5 mmol) of 2′,3′,5′-tri-*O*-acetylguanosine (**15**) in 10 ml of freshly distilled anhydrous pyridine was added 1.63 g (5.0 mmol) of the isocyanate of *O*-benzyl-L-threonine benzyl ester (Chheda and Hong, 1971) and the mixture heated with stirring at 90° for 6 hr in a glass bomb. The solution was then evaporated to dryness and the residue was azeotroped twice with 10 ml of toluene. The residue contained unchanged tri-*O*-acetylguanosine, the reaction product (**16**), and a fast moving material. It was dissolved in 15 ml of $CHCl_3$ and adsorbed on the top of a column of silica gel (100–200 mesh, dry packed, 2.54 × 68 cm). The column was eluted first with 500 ml of $CHCl_3$ and then with 1200 ml of $CHCl_3$–MeOH (19:1). The protected product, *N*-[9-(2′,3′,5′-tri-*O*-acetyl-β-D-ribofuranosyl)-1*H*-6-oxopurin-2-ylcarbamoyl]-*O*-benzyl-L-threonine benzyl ester, was eluted out between 500 and 1200 ml of $CHCl_3$–MeOH (19:1). The solvent was evaporated to dryness to yield 646 mg (34.7%) of a gummy product which was found homogeneous by TLC in solvents B, C, and E. Anal. Calcd for $C_{35}H_{38}N_6O_{12} \cdot 0.5H_2O$: C, 56.53; H, 5.25; N, 11.30. Found: C, 56.49; H, 5.44; N, 11.03.

N-[(*9-β*-D-*Ribofuranosyl-1H-6-oxopurin-2-yl*)*carbamoyl*]-L-*threonine* (*t*^{2}*G*, **18**). The gummy compound **16** (600 mg) was dissolved in 30 ml of MeOH and hydrogenated under atmospheric pressure and at room temperature with 250 mg of PdO until there was no further consumption of H_2 (12 hr). The catalyst was filtered and the solvent was evaporated to dryness. The residue was stirred in 20 ml of 4 *N* NH_3–MeOH at 4° for 6 hr and the precipitated white solid was collected on a filter and washed with MeOH. The crude material was crystallized twice from absolute EtOH: yield 305 mg (83.1%); mp 203–204° dec; ir max (cm^{-1}) 1700 (ureido C=O), 1600, and 1530 (C=C, C=N); NMR δ (Me_2SO-d_6) 1.03 (d, 3, J = 6 Hz, $-CHCH_3$), 3.40–4.50 (m, 7, 3′-H, α and β-C*H*, 2′-H, 4′-H, 5′-H), 5.83 (d, 1, J = 6 Hz, 1′-H), 7.99 (broad, 1, $-NH$), and 8.18 ppm (s, 1, 8-H). Anal. Calcd for $C_{15}H_{20}N_6O_9 \cdot 1.5H_2O$: C, 39.56; H, 5.05; N, 18.46. Found: C, 39.29; H, 5.12; N, 18.69.

N-[(*9-β*-D-*Ribofuranosyl-1H-6-oxopurin-2-yl*)*carbamoyl*]*glycine* (*g*^{2}*G*, **19**). To a solution of 1.02 g (2.5 mmol) of 2′,3′,5′-tri-*O*-acetylguanosine in 10 ml of anhydrous pyridine was added 0.955 g (5.0 mmol) of the isocyanate of glycine benzyl ester and the mixture was heated with stirring at 90° for 6 hr in a glass bomb. The mixture was worked up in the same way as described for **16.** The protected benzyl ester derivative (**17**) was hydrogenolyzed and deacetylated in the same way as **16.** The product (**19**) was cystallized twice from warm water: yield 238 mg (23.7%); mp >220° dec; ir max (cm^{-1}) 1700 (ureido C=O), 1610, and 1530 (C=C, C=N); NMR δ (Me_2SO-d_6) 3.92 (d, 2, J = 4 Hz, $-NHCH_2-$), 3.40–4.60 (m, 5, 2-H, 3′-H, 4′-H, 5′-H), 5.80 (d, 1, J = 6 Hz, 1′-H), 7.18 (m, 1, $-NH$), 8.17 (s, 1, 8-H), 10.33 (broad s, 1, $-NH$), and 11.87 ppm (m, 1, $-NH$). Anal. Calcd for $C_{13}H_{16}N_6O_8 \cdot H_2O$: C, 38.80; H,

4.48, N, 20.90. Found: C, 38.95; H, 4.43; N, 20.93.

2′,3′,5′-Tri-O-acetylcytidine (**20**). A mixture of 2.43 g (10.0 mmol) of cytidine and 6 ml of acetic anhydride in 20 ml of anhydrous pyridine was stirred at room temperature for 16 hr. (Solution occurred in 30 min.) 10 ml of MeOH was added to the reaction mixture which was then evaporated to dryness in vacuo (ca. 50°). The last traces of pyridine were removed by azeotroping three times with toluene (10 ml). The residue N^4,2′,3′,5′-tetraacetylcytidine was obtained as a solid foam, yield 4.0 g (97.3%). The material was homogeneous by TLC in solvents B, C, and E and no cytidine or partially acetylated cytidine could be detected.

The above tetraacetylcytidine was dissolved in 100 ml of a mixture of $CHCl_3$ and MeOH (65:35) containing 9.7 ml of aqueous 1 *N* HCl. The mixture was allowed to stand at room temperature for 26 hr. The course of hydrolysis of the N^4-acetyl group was followed by TLC in a $CHCl_3$–MeOH (19:1 v/v) solvent system. TLC after 26 hr showed a faint spot for tetraacetylcytidine and a major spot, as checked by uv, presumably due to 2′,3′,5′-tri-*O*-acetylcytidine. The reaction mixture was then evaporated to dryness in vacuo at 35°. To remove the last traces of HCl and water, the residue was dissolved in 25 ml of a mixture of EtOH–benzene (1:1) and evaporated to dryness at the same temperature. The procedure was repeated five times and finally the residue was dried under high vacuum over KOH at 35° for 1 hr and then at 60° for 2 hr. The crude foam was dissolved in 500 ml of hot EtOAc; the solution was filtered, concentrated to a small volume, and cooled at 0° to give a semisolid product which was filtered and washed with EtOAc. This was dissolved in 20 ml of MeOH and reprecipitated to give a gelatinous precipitate by addition of 500 ml of ether. This process was repeated twice and the fine powder was collected on a filter and air dried, yield 3.4 g (92.1%), mp 165–168°.

N-[(*1-β*-D-*Ribofuranosyl-2-oxopyrimidin-4-yl*)*carbamoyl*]-L-*threonine* (*t⁴C*, **23**). To a solution of 0.923 g (2.5 mmol) of 2′,3′,5′-tri-*O*-acetylcytidine in anhydrous pyridine (12 ml) was added 1.63 g (5.0 mmol) of isocyanate of *O*-benzyl-L-threonine benzyl ester. The mixture was heated with stirring at 90° for 6 hr in a glass bomb and then evaporated to dryness. The residue was azeotroped twice with 20 ml of toluene. The residue contained the reaction product (**21**), some unchanged tri-*O*-acetylcytidine, and a fast moving material. The mixture was separated by preparative TLC (6 plates, 0.15 × 20 × 100 cm) in solvent B and the product **21** was extracted from the silica gel with boiling $CHCl_3$ (10 × 200 ml). The solvent was removed by evaporation to yield a gummy product (0.501 g, 28.9%). It was characterized by uv spectra (λ_{max} 287 and 245 nm in H_2O) and by TLC in solvents B, C, and E. This material was dissolved in 20 ml of absolute EtOH and then hydrogenated with 200 mg of PdO under atmospheric pressure and at room temperature for a period of 16 hr. The catalyst was filtered and the solvent was evaporated. The gummy residue was dissolved in 15 ml of 4 *N* NH_3–MeOH and stirred at 4° for 6 hr. The precipitated white solid was filtered, washed with MeOH, and crystallized three times from a mixture of MeOH–EtOAc (1:1): yield 147 mg (48.0%); mp 197–200°; ir max (cm^{-1}) 1700 (ureido C=O), 1630, and 1550 (C=C, C=N). Anal. Calcd for $C_{14}H_{20}N_4O_9 \cdot 2H_2O$: C, 39.60; H, 5.65; N, 13.20. Found: C, 39.77; H, 5.45; N, 13.17.

N-[(*1-β*-D-*Ribofuranosyl-2-oxopyrimidin-4-yl*)*carbamoyl*]*glycine* (*g⁴C*, **24**). To a solution of 0.923 g (2.5 mmol) of 2′,3′,5′-tri-*O*-acetylcytidine in 10 ml of anhydrous pyridine was added 0.955 g (5.0 mmol) of isocyanate of glycine benzyl ester and the mixture was heated with stirring at 90° for 6 hr in a glass bomb. The reaction mixture was worked up in the same manner as described for **23** and the product was crystallized twice from absolute EtOH, yield 210 mg (25.5%): mp 200–201° dec; ir max (cm^{-1}) 1700 (ureido C=O), 1620, and 1560 (C=C, C=N); NMR δ (Me_2SO-d_6) 3.80 (d, 2, J = 5 Hz, $-NHCH_2-$), 3.60–4.50 (m, 5, 3′-H, 2′-H, 4′-H, 5′-H), 5.78 (d, 1, J = 4 Hz, 1′-H), 6.4 (d, 1, J = 8 Hz, 5′-H), 7.20 (broad, 1, $-NH$), 8.23 (d, 1, J = 7 Hz, 6-H), and 8.87 ppm (broad, 1, $-NH$). Anal. Calcd for $C_{12}H_{16}N_4O_8 \cdot H_2O$: C, 39.77; H, 4.97; N, 15.47. Found: C, 39.79; H, 4.95; N, 15.53.

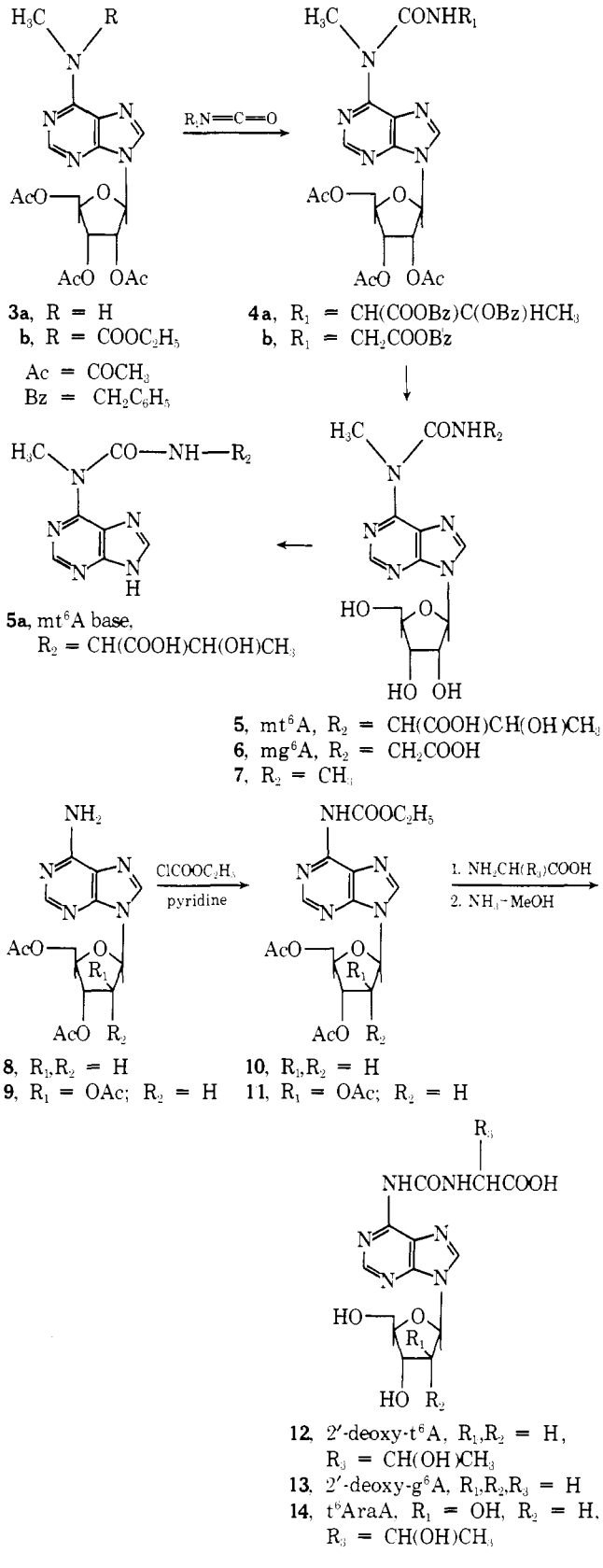
Incubation of mt⁶A with Enzymes. To 2.9 ml of a solution of mt⁶A in an appropriate buffer (13.0 mg/l.) was added 0.1 ml of enzyme (1 mg) in the same buffer and the mixture was incubated at room temperature. Uv spectra (350–220 nm) were recorded just before and after addition of the enzyme, and then after 2, 6, and 20 hr. A solution of the enzyme in buffer at the same concentration was used as the blank. The uv spectra of mt⁶A did not show any change after incubation with the enzyme. The following enzymes were used under proper pH and incubation conditions (Colowick and Kaplan, 1955, 1957): urease (jack bean) (0.75 *M* phosphate, pH 7.0), peptidase (hog intestine) (0.08 *M* Tris, pH 7.5), and protease (P-K) (0.08 *M* Tris, pH 7.8).

Results

Attempts to convert t⁶A (**1**) to mt⁶A (**5**) by direct methylation with CH_3I, and with formaldehyde and $NaBH_3CN$ (Borch and Hassid, 1972), were not successful. In order to apply the urethane procedure of Chheda and Hong (1971) for the synthesis of mt⁶A, attempts were made to prepare the 6-carbamate (**3b**). Methylation of ethyl 9-(2′,3′,5′-tri-*O*-acetyl-*β*-D-ribofuranosyl)purine-6-carbamate (Chheda and Hong, 1971) with CH_3I and with diazomethane failed to give a significant yield of the methylated product. Acylation of the tri-*O*-acetyl-N^6-methyladenosine (**3a**) with ethyl chloroformate in pyridine to give the desired carbamate (**3b**) also proved futile. Thus, for the synthesis of *N*-[(9-*β*-D-ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]-L-threonine (mt⁶A) (**5**) from N^6-methyladenosine, the isocyanate method was used (Scheme I). N^6-Methyladenosine was converted into 2′,3′,5′-tri-*O*-acetyl-N^6-methyladenosine (**3a**) by acetic anhydride in pyridine. This acetylated compound, when allowed to react with the isocyanate of *O*-benzyl-L-threonine benzyl ester (Chheda and Hong, 1971) in hot pyridine, gave the fully protected derivative of mt⁶A (**4a**) in good yield. Hydrogenation of **4a** over PdO followed by the treatment with methanolic ammonia gave the desired product, mt⁶A. The corresponding glycine derivative *N*-[(9-*β*-D-ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]glycine (mg⁶A, **6**) was prepared by the reaction of the isocyanate derived from glycine benzyl ester with 2′,3′,5′-tri-*O*-acetyl-N^6-methyladenosine followed by the removal of the protecting groups. The methyl ureido analog **7** was also prepared in a similar manner.

For the preparation of t⁶A analogs from 2′-deoxyadenosine and 9-(*β*-D-arabinofuranosyl)adenine, the urethane method was applied (Chheda and Hong, 1971) (Scheme I). 3′,5′-Di-*O*-acetyl-2′-deoxyadenosine (**8**) was prepared by a reaction of 2′-deoxyadenosine with acetic anhydride in pyridine at room temperature(Hayes et al., 1955). Reaction of ethyl chloroformate with the diacetyl-2′-deoxyadenosine (**8**)

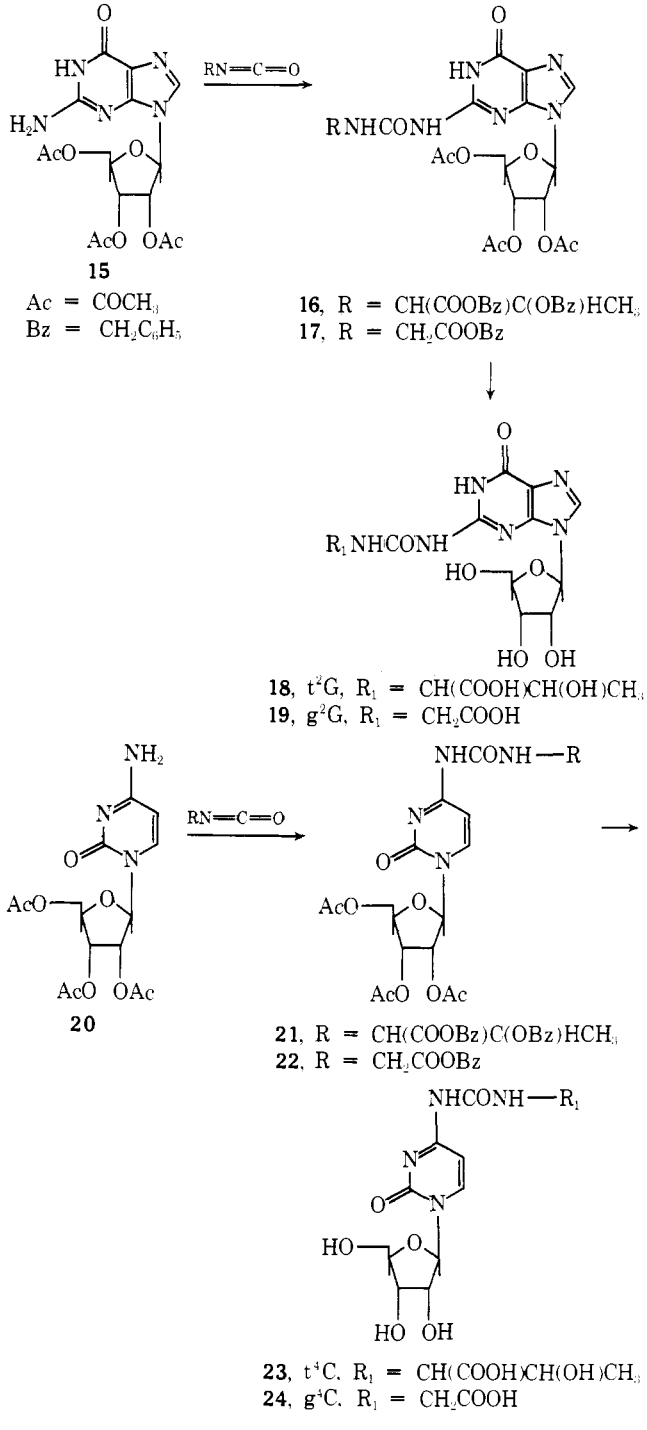
Scheme I



in pyridine at −10° gave ethyl 9-(3′,5′-di-*O*-acetyl-β-D-2′-deoxyribofuranosyl)purine-6-carbamate (**10**) in 43% yield. The displacement of the ethoxy group of the urethane (**10**) by L-threonine and glycine in hot pyridine followed by the removal of acetyl groups gave the corresponding 6-ureidopurine deoxyribonucleosides, 2′-deoxy-t^{6}A (**12**) and 2′-deoxy-g^{6}A (**13**). Ethyl 9-(2′,3′,5′-tri-*O*-acetyl-β-D-arabinofuranosyl)purine-6-carbamate (**11**) was prepared by a reaction of tri-*O*-acetylarabinosyladenine (**9**) and ethyl chloroformate in pyridine. This urethane was then converted into the *N*-[(9-β-D-arabinofuranosylpurin-6-yl)carbamoyl]-L-threonine (t^{6}AraA, **14**) by our usual reaction procedures as discussed above.

The compounds corresponding to t^{6}A and g^{6}A were also prepared from guanosine and cytidine (Scheme II). The reaction of 2′,3′,5′-tri-*O*-acetylguanosine with the isocyanates of *O*-benzyl-L-threonine benzyl ester and glycine benzyl ester in hot pyridine gave the corresponding fully protected N^2-carbamoyl derivatives **16** and **17**, respectively. These compounds upon hydrogenation followed by deacety-

Scheme II



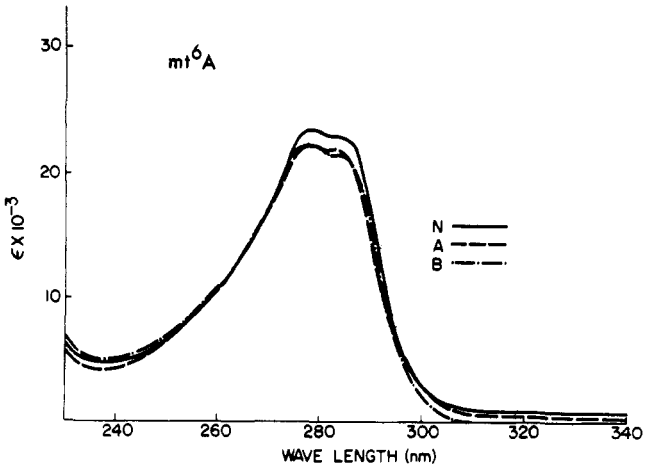


Fig. 1A

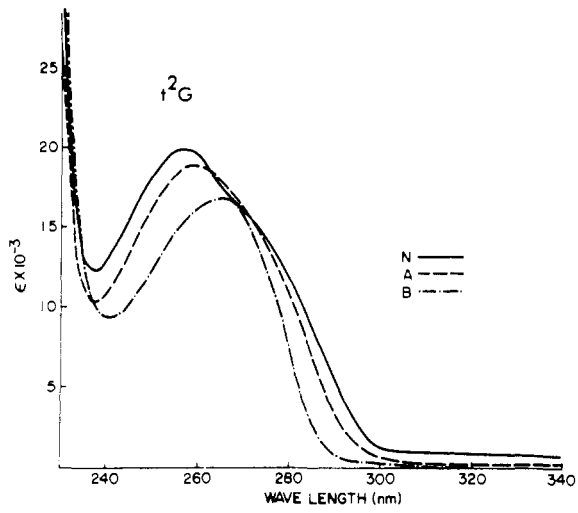


Fig. 1B

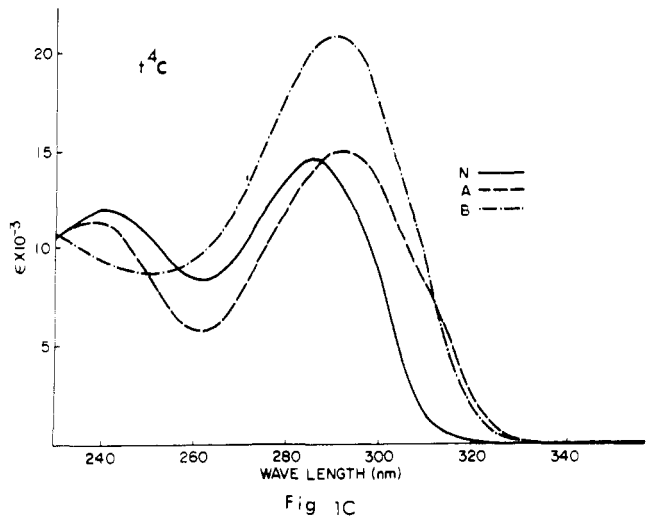


Fig 1C

FIGURE 1: Quantitative uv spectra of mt^{6}A (A), t^{2}G (B), and t^{4}C (C): N (—), H_2O; A (- - -), 0.1 *N* HCl; B (···), 0.1 *N* NaOH.

lation gave the desired products, *N*-[(9-β-D-ribofuranosyl-1*H*-6-oxopurin-2-yl)carbamoyl]-L-threonine (t^{2}G, **18**) and *N*-[(9-β-D-ribofuranosyl-1*H*-6-oxopurin-2-yl)carbamoylglycine (g^{2}G, **19**), respectively. For the preparation of cytidine analogs of t^{6}A and g^{6}A, we needed a suitably protected 2′,3′,5′-tri-*O*-acetylcytidine (**20**). Preparation of the latter compound (**20**) has been described in the literature (Beranek and Pitha, 1964). However, attempts to adapt this procedure to a large scale preparation of 2′,3′,5′-tri-*O*-acetylcytidine were unsuccessful. We have now prepared this compound in large amounts by selective N-deacylation of tetraacetylcytidine (Beranek and Pitha, 1964) with dilute HCl at room temperature (Goody and Walker, 1971). Reaction of the 2′,3′,5′-tri-*O*-acetylcytidine with the isocyanates of *O*-benzyl-L-threonine benzyl ester and glycine benzyl ester, and subsequent debenzylation and deacetylation, furnished the desired compounds, *N*-[(1-β-D-ribofuranosyl-2-oxopyrimidin-4-yl)carbamoyl]-L-threonine (t^{4}C, **23**) and *N*-[(1-β-D-ribofuranosyl-2-oxopyrimidin-4-yl)carbamoyl]glycine (g^{4}C, **24**), respectively (Chheda et al., 1974).

The t^{6}A (**1**) and g^{6}A (**2**) have the uv maxima at 270 and 277 nm in neutral solution, while both mt^{6}A (**5**) and mg^{6}A (**6**) have two ultraviolet absorption maxima at 278 and 284 nm (Figure 1A). Unlike t^{6}A (**1**) and g^{6}A (**2**), however, these maxima do not exhibit any shift in acidic or basic pH (Fig. 1A). The mt^{6}A was stable at room temperature in neutral (pH 5.5) and acidic media (pH 2). In 0.1 *N* NaOH solution as well as in hot water mt^{6}A underwent degradation to give *N*^{6}-methyladenosine and threonine. The ultraviolet and NMR spectra are in agreement with the assigned structure of mt^{6}A (Kimura-Harada et al., 1972). These results support the structure of mt^{6}A in terms of the substitution of *N*^{6}-nitrogen by both the methyl and by the carbamoyl group. Treatment of mt^{6}A by 1 *N* HCl at room temperature for 60 hr gave the free base *N*-(purin-6-yl-*N*-methylcarbamoyl)-L-threonine (**5a**) as a major product as identified from its uv spectra (λ_{max} water 280, 0.1 *N* HCl 282, 0.1 *N* NaOH 283 nm). The t^{6}A analogs of 2′-deoxyadenosine (**12**) and 9-(β-D-arabinofuranosyl)adenine (**14**) have uv spectra similar to t^{6}A in acidic, neutral, and basic pH. The structures of the quanosine analogs t^{2}G (**18**) and g^{2}G (**19**) were assigned on the basis of acid hydrolysis of **18** to give a product, which had an uv spectrum similar to that of *N*^{2}-acetylguanine (Shapiro et al., 1969) and *N*^{2}-carbamoylguanine (S. P. Dutta et al., unpublished results). The guanosine derivatives (**18** and **19**) showed an uv absorption maxima at 256 nm in neutral aqueous solution which shifted to 258 and 265 nm in acidic and basic pH, respectively (Figure 1B). The structures of the cytidine derivatives t^{4}C and g^{4}C (**23** and **24**) were assigned on the basis of their stability and similarity of their uv spectra with *N*^{4}-acetylcytidine (Watanabe and Fox, 1966). The cytidine compounds t^{4}C and g^{4}C (**23** and **24**) have absorption maxima at 286 and 242 nm in aqueous solution which shifted to 293 and 240 nm in acidic pH and 292 nm in basic pH (Figure 1C). This could be compared to the uv spectrum of *N*^{4}-acetylcytidine which had uv absorption maxima at 294 and 245 nm in aqueous solution.

Discussion

This study presents a chemical synthesis of the naturally occurring nucleoside mt^{6}A. The mt^{6}A, isolated from *E. coli* tRNA^{Thr}, was found to be identical with our synthetic mt^{6}A (S. Nishimura, personal communication) in TLC and in paper chromatography in four solvent systems as well as in its uv spectral properties. The natural mt^{6}A isolated from the wheat embryo tRNA and from rat liver tRNA was also identical with the synthetic mt^{6}A in paper chromatography

and electrophoresis, as well as in its uv spectra (Gray, 1974; Rogg et al., 1975). In addition to the synthesis of mt^{6}A, this study provides a number of carbamoylthreonine and carbamoylglycine containing guanosine analogs (t^{2}G and g^{2}G), cytidine analogs (t^{4}C and g^{4}C), and 2′-deoxyadenosine analogs (2′-deoxy-t^{6}A and 2′-deoxy-g^{6}A). These compounds so far have not been found as components of nucleic acids but can be considered of potential biological significance in terms of their possible natural occurrence.

The metabolic fate of mt^{6}A was very similar to that of t^{6}A (Hong et al., 1973b). Like t^{6}A, when 8-^{14}C-labeled mt^{6}A was administered intravenously to rat, 80% of the radioactivity was excreted in urine in 48 hr as an unchanged material (Chheda et al., 1973). From these results, it is suggested that mt^{6}A is a metabolically stable modified nucleoside. Since no radioactivity could be detected from the rat liver tRNA after administration of mt^{6}A-*8*-^{14}*C* and *N*^{6}-methyladenosine-*8*-^{14}*C*, it is suggested that the mt^{6}A cannot be incorporated into the tRNA (unpublished results) and it appears that the biosynthesis of the latter occurs at a macromolecular level. Though t^{6}A could not be methylated chemically to mt^{6}A in reasonable yield, biochemically it may still serve as a substrate for methylation by *S*-adenosylmethionine. Orally administered 8-^{14}C-labeled mt^{6}A was not absorbed, but was excreted in feces. In vitro, mt^{6}A was not hydrolyzed by urease, protease, and peptidase. The mt^{6}A, mg^{6}A, and other analogs did not show any growth inhibitory activity toward mouse leukemic cells L-1210 and human leukemic cells RPMI 6410 grown in culture.

Although the functions of all the modified nucleosides are not fully understood, it is obvious that the anticodon adjacent modified nucleosides must play some role in the overall process of protein biosynthesis. From the conformational studies we have earlier suggested that one of the functions of the anticodon adjacent nucleosides may be to prevent the misreading of the codons by their inability to participate in Watson–Crick base pairing with the bases on the mRNA (Parthasarathy et al., 1974). The X-ray crystallographic studies on t^{6}A and the NMR solution studies on t^{6}A base show that the α-NH of threonine is hydrogen bonded to the N^1 of adenine and that the threonine side chain is distal (trans) to the imidazole portion of the molecule. In mt^{6}A the presence of an N^6-methyl group perhaps doubly ensures that there can be no Watson–Crick base pairing between mt^{6}A in tRNA and uracil in mRNA. The studies related to the hydrogen bonding abilities of mt^{6}A for base pairing are in progress.

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