NUCLEIC ACID RELATED COMPOUNDS. 20. SUGAR, BASE DOUBLY MODIFIED NUCLEOSIDES AT THE 5'-TERMINAL "CAP" OF mRNAs AND IN NUCLEAR RNA.

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SUMMARY: N^6,$O^{2'}$-Dimethyladenosine (III) has recently been reported to occur adjacent to the modified guanosine at the 5'-terminal "cap" of a number of mRNAs and in nuclear RNA. The possible presence of N^6,N^6,$O^{2'}$-trimethyladenosine (V) in adenovirus mRNA "caps" has been suggested on the basis of labeled methyl distribution between sugar and base fractions. Methylation of 2'-O-methyladenosine (I) gave N^1,$O^{2'}$-dimethyladenosine hydroiodide (II) which was rearranged to give III. Sugar methylation of 6-chloropurine riboside (IV) followed by replacement of chloro with dimethylamine gave V plus its $O^{3'}$-isomer (VI). Thin layer chromatography systems which cleanly resolve II, III, and V have been devised. Adenosine aminohydrolase (E.C. 3.5.4.4) deaminates III slowly, but has no effect on V. Identifying spectral data are tabulated.

The 5'-termini of a variety of mRNAs have been found to be "capped" by the sequences m^7G5'-p(p)$_{0-1}$p-5'Nm(pNm)$_{0-X}$* (1). The biological role of these unusual 5'-termini is unknown, but the highly modified sequence appears to be required for effective translation (2). Resistance toward enzymic cleavage of internucleotide bonds involving 2'-O-methylated units has been noted (3), and a protective function accruing during maturation has been considered (1b). The structure of the 5'-penultimate nucleoside occurring in certain animal cell and virus mRNAs was reported to be N^6,$O^{2'}$-dimethyladenosine (III) (4). This assignment was based on chromatographic and electrophoretic comparisons with previously known N^6 and $O^{2'}$ monomethyl components plus its acid catalyzed hydrolysis or enzymic phosphorolysis to compounds which chromatographed with N^6-methyladenine or 2-O-methylribose-1-phosphate (4). The doubly modified nucleoside (III) has also been detected in an interior region of nuclear RNA

* Abbreviations: m^7G, 7-methylguanosine; Nm, a 2'-O-methylnucleoside.

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from tumor sources (5), and an unidentified nucleoside which was presumed to be a base modified derivative of 2'-O-methyladenosine (I) was found adjacent to m^7G in mouse and viral mRNAs (6). Acid catalyzed hydrolysis of the "cap" nucleosides from ^3H-methyl labeled adenovirus mRNAs was very recently found to give a higher distribution of radioactivity in the base fraction (relative to the 2-O-methyl sugar), and the possible presence of N^6,N^6,$O^{2'}$-trimethyladenosine (V) was suggested (7).

We wish to report convenient syntheses of III and its N^1-isomer (II) from I (see Figure I) and V plus its $O^{3'}$-isomer (VI) from 6-chloropurine riboside (IV) (see Figure II). Thin layer chromatography (tlc) systems have been devised which cleanly separate II, III, and V and a simple enzyme assay employing adenosine aminohydrolase (E.C. 3.5.4.4) readily distinguishes III from V. ^1H nmr and mass spectral data easily identify each of these methylated adenosine nucleosides if sufficient material is available.

MATERIALS AND METHODS

Melting points were determined on a Reichert microstage apparatus. Ultraviolet spectra were recorded on a Pye-Unicam SP1700 instrument. Optical rotations (1-ml, 10-cm microcell) were measured on a Perkin-Elmer 241 polarimeter. ^1H nmr spectra (Me_2SO-d_6, TMS internal) were determined on a Varian HA-100 instrument. Mass spectra (70 eV, ~250° direct probe) were obtained by the mass spectroscopy laboratory on an AEI MS-50 with computer averaging. Adsorption (J. T. Baker silica gel no. 3405) and ion-exchange (8) (Dowex 1-X2[OH^-] 200-400 mesh) column chromatography; paper (A [ascending, n-BuOH—conc. NH_4OH—H_2O 86:5:14] and B [descending, i-PrOH—conc. NH_4OH—H_2O 7:1:2] on Whatman no. 1) chromatography; and tlc (Eastman silica gel sheet no. 13181 with no. 6060 indicator, C [MeOH—$CHCl_3$ 1:9]) were performed in the usual manner. All solvents and reagents were of commercial reagent purity and solvents were distilled before use. The buffer solution used was 0.05 M KH_2PO_4—NaOH (pH 7.0). Adenosine deaminase type I (lot no. 121C-0460) was purchased from Sigma Chemical Co., St. Louis, and 6-chloropurine riboside (IV) was purchased from Terochem Laboratories Ltd., Edmonton. Compound I was prepared as described (9).

N^1,$O^{2'}$-Dimethyladenosine Hydroiodide (II): To 800 mg (2.85 mmole) of I in 10 ml of N,N-dimethylacetamide (DMAC) was added 1 ml of methyl iodide and the resulting solution was stirred for 20 hr at room temperature in the dark. Tlc (C) indicated complete conversion of I to II. Celite (300 mg) was added and the stirred mixture was filtered. The filter cake was washed with 10 ml of acetone and the combined filtrate was placed in a closed desiccator containing ether for 18 hr. The resulting white crystalline solid was filtered and washed well with ether to give 1.06 g (88%) of II, mp 221-222° (dec); $[\alpha]_D^{27}$ - 23.3° (c 1, H_2O), -24.2° (c 1, pH 7.0 buffer). Anal. Calcd for $C_{12}H_{17}N_5O_4 \cdot HI$: C, 34.05; H, 4.29; N, 16.55; I, 29.99. Found: C, 33.82; H, 4.26; N, 16.58; I. 29.86.

N^6,$O^{2'}$-Dimethyladenosine (III): To 500 mg (1.18 mmole) of II was added 50 ml of dimethylamine—methanol (1:1) and the resulting solution was stirred for 18 hr at room temperature in the dark. Tlc (C) indicated complete conversion of II to III. The solution was evaporated *in vacuo*, the residue was dissolved in acetone, 3 g of silica gel was added, and the mixture was evaporated to dryness. The impregnated adsorbant was applied to a column (2.5 x 24 cm, 50 g) of silica gel which was then washed with 275 ml of chloroform and the wash was discarded. Elution (methanol—chloroform 1:9) and evaporation of appropriate uv absorbing fractions gave 357 mg (quantitative) of tlc (C) homogeneous product. One portion of this product was dissolved in acetone and precipitated into n-pentane—ether (1:1). A second portion was dissolved in ethanol and treated with ethanolic HCl to give 88% yield of the crystalline hydrochloride salt. III·HCl had mp 179-180°; $[\alpha]_D^{27}$ -34.6° (c 0.9, H_2O), -48.9° (c 1, pH 7.0 buffer). Anal. Calcd for $C_{12}H_{17}N_5O_4 \cdot HCl$: C, 43.44; H, 5.47; N, 21.11; Cl, 10.69. Found: C, 43.67; H, 5.58; N, 20.84; Cl, 10.78.

N^6,N^6,$O^{2'}$-Trimethyladenosine (V) and N^6,N^6,$O^{3'}$-Trimethyladenosine (VI): To 750 mg (2.62 mmole) of IV suspended in 155 ml of 98% ethanol containing 39 mg of stannous chloride dihydrate was added diazomethane in 1,2-dimethoxyethane as described previously (9). After the yellow color of diazomethane persisted, tlc (C) indicated complete conversion of IV to faster migrating products. The solution was evaporated and the residue was stirred for 20 min at room temperature with 25 ml of anhydrous dimethylamine. Tlc (C) indicated complete reaction after 5 min. The solution was evaporated and the residue was dissolved in water and applied to a column (2 x 59 cm) of anion exchange resin packed in water. Elution with water followed by 10% stepwise increments of ethanol in water effected clean separation of V (34%) from VI (55%) and unidentified permethylated products (4%). Purification of V from a small (<3%) quantity of contaminating N^6,N^6,$O^{2'}$,$O^{5'}$-tetramethyladenosine (^1H nmr and mass spectra) was effected by thick layer chromatography (Merck silica gel PF 254, ethanol—chloroform 4:96, developed four times). The resulting homogeneous product was crystallized from isopropyl alcohol/ether/n-pentane to give large cubic crystals of V, mp 123-125°; $[\alpha]_D^{27}$ -65.5° (c 1, MeOH); mass spectrum m/e (% relative intensity, ion) 309.1435(22, M^+ 309.1437), 279(12, M - 15), 220(90, BHCH=CHOCH$_3$), 192(61, BHCHO), 164(92, B + 2H), 163(87, B + H), 146(23, sugar ion "w" [9,10]), 134(100, BH - CH_2=NH). Anal. Calcd for $C_{13}H_{19}N_5O_4$: C, 50.48; H, 6.19; N, 22.64. Found: C, 50.60; H, 6.25; N, 23.06.

Crystallization of VI from 98% ethanol gave fine needles, mp 169-171°; $[\alpha]_D^{27}$ -70.5° (c 1, MeOH); mass spectrum 309.1432(13, M^+ 309.1436), 279(8, M - 15), 206(19, BHCH=CHOH), 192(43, BHCHO), 164(58, B + 2H), 163(36, B + H), 134(100, BH - CH_2=NH). Anal. Found: C, 50.21; H, 6.10; N, 22.90.

Enzymic Deamination: Solutions (~5 x 10^{-5} M) of adenosine, I, III, and V were treated with 25 fold (~0.23 units/ml) and 50 fold (~0.46 units/ml) the assay concentration of adenosine deaminase described previously (11). Uv absorption profiles were monitored periodically. At 25 fold enzyme concentration both adenosine and I were completely deaminated in <1 min. Compound III was totally converted to the hypoxanthine product in <24 hr (25 fold) or <15 hr (50 fold), but V remained unchanged after 64 hr in the presence of 25 or 50 fold enzyme.

RESULTS AND DISCUSSION

Methylation of I (9) (see Figure I) by the procedure of Jones and Robins (12) gave II in good yield. Dimroth rearrangement of II to III proceeded readily in methanolic dimethylamine (13). Compound III resisted crystalliza-

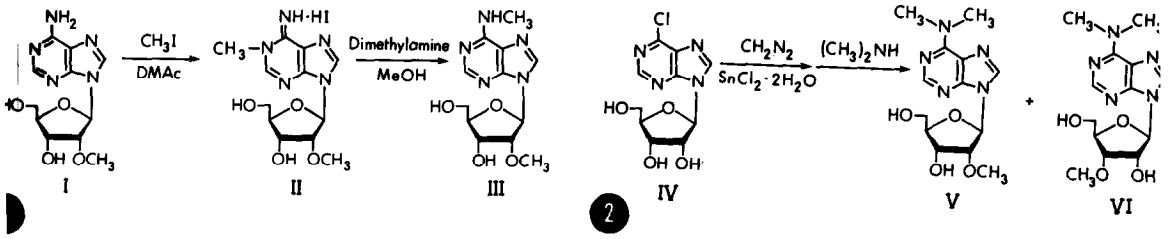

FIGURE I. Synthesis of N^1,$O^{2'}$-dimethyladenosine (II) and N^6,$O^{2'}$-dimethyladenosine (III).

FIGURE II. Synthesis of N^6,N^6,$O^{2'}$-trimethyladenosine (V) and N^6,N^6,$O^{3'}$-trimethyladenosine (VI).

tion and was converted to its crystalline hydrochloride salt for analysis. This material had the same mass spectrum (vaporization of HCl before fragmentation) as a sample of the amorphous free base purified by precipitation. Since the optical rotation of a nucleoside is markedly sensitive to pH in the region of its pKa, the value for III·HCl was determined in pH 7.0 buffer as well as in water.

Selective sugar methylation of IV (see Figure II) using diazomethane with stannous chloride dihydrate as catalyst gave the $O^{2'}$ and $O^{3'}$ methyl isomers as major products (9). However, over-methylation of sugar hydroxyls was observed with IV (in contrast to the clean monomethylation of adenosine [9]). Treatment of the crude reaction product with dimethylamine gave V and VI in 89% combined yield plus ~7% of more highly methylated derivatives. The desired V was cleanly separated from VI using anion exchange chromatography (8) and purified from ~3% of its $O^{5'}$-methyl derivative by thick layer chromatography. ^{1}H nmr (Table 1) and ultraviolet (Table 2) spectral data are in accord with assigned structures.

Mass spectral fragmentation of III (see Figure III B) occurs in accordance with previous studies on methylated adenosine derivatives (9,10). Heterocyclic base + H (m/e 149) is the mass spectral base peak (100% relative intensity). The 2'-identifying ion (BHCH=CHOCH$_3$) (m/e 206, 68%); (BHCHO) (m/e 178, 49%); (B + 2H) (m/e 150, 89%); the 2-O-methyl sugar fragment (ion "w"

TABLE 1. ^{1}H nmr Spectral Data[a]

Compound	$H^{5'}$,$H^{5''}$[b]	$H^{4'}$[b]	$H^{3'}$	$H^{2'}$	$H^{1'}$[c]	H^{2}[d]	H^{8}[d]	OCH_3[d]	$N(CH_3)$
II	3.65	4.03	(4.34)[b]		6.07	(8.73,8.79)		3.36	3.81[d]
III	3.65	4.02	(4.38)[b]		6.04	8.25	8.39	3.32	3.03[e]
V	3.62	3.98	(4.34)[b]		6.03	8.22	8.40	3.32	3.45[d]
IV	3.64	4.08	3.88	4.75	5.93	8.23	8.38	3.43	3.47[d]

[a] In Me_2SO-d_6, δ ppm from internal Me_4Si. [b] Multiplets. [c] Doublets, $J_{1'-2'}$ = 5.0-6.0 Hz. [d] Singlets. [e] Doublet, J_{CH_3-NH} = 3.7 Hz.

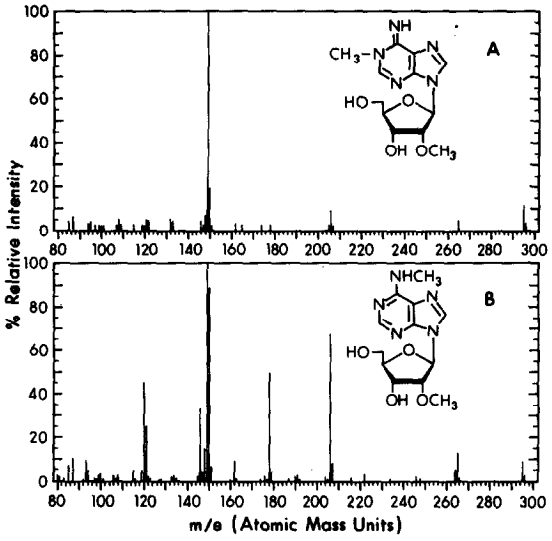

FIGURE III. Mass spectra (m/e 80 to 300) of compounds II (A) and III (B) under identical conditions.

[9,10]) (m/e 146,33%); and (BH - CH_2=NH, $C_5H_4N_4$: 120.0436) (observed m/e 120.0436, 46%) plus its protonated form ($C_5H_5N_4$: 121.0514) (observed m/e 121.0507, 26%) are the major fragment ions. Vaporization of HI again occurs before fragmentation of II (see Figure III A) in the mass spectrometer. Only (B + H) (m/e 149, 100%); (B + 2H) (m/e 150, 19%); (M^+) (m/e 295, 12%); and

TABLE 2. Ultraviolet Absorption and Paper Chromatography Data

Compound	UV Spectral Data[a]				Chromatographic Mobilities[b]	
	0.1 N HCl		0.1 N NaOH			
	max	min	max	min	A	B
II	258(14,200)	243(10,500)	259(13,100) 266(11,700) shoulder[c]	244(9000)	0.48	0.65
III	263(15,900)	232(2800)	266(15,200)	231(2200)	0.69	0.75
V	268(17,800)	235(2800)	276(18,400)	239(2800)	0.71	0.77
VI	268(16,800)	235(2800)	275(17,100)	240(2600)	0.74	0.77

[a] Maxima and minima in nm(ε). [b] See Materials and Methods for conditions.
[c] Acidification of this solution gave a spectrum superimposible with the original acid spectrum.

($BHCH{=}CHOCH_3$) (m/e 206, 10%) are of ≥10% relative intensity in this mass spectrum. Identifying fragment ions in the mass spectra of V (10) and VI are given in Materials and Methods.

It is of interest that the basic chromatographic solvents (4) used in the identification and characterization of III do not cause rearrangement of II to III (see Table 2). Direct comparison of III and the product obtained from HeLa cell mRNA (4) in paper chromatographic systems A and B showed identical mobilities (Dr. B. Moss, private communication). This suggests that the N^6-derivative (III) and not its N^1 counterpart (II) is the naturally occurring isomer. However, as seen in Table 2, the paper chromatographic mobilities of III and V in systems (4) A and B are nearly equal and could not be expected to distinguish these di (4) and tri (7) methyladenosines. Tlc using ethyl acetate—acetone (1:1) cleanly resolves II, III, and V (see Figure IV). Cyclohexane—acetone (1:1) and benzene—acetone (1:1) are equally effective solvent systems on silica gel tlc plates for separating III and V (as well as II).

Suhadolnik (14) had noted that adenosine aminohydrolase (E.C. 3.5.4.4)

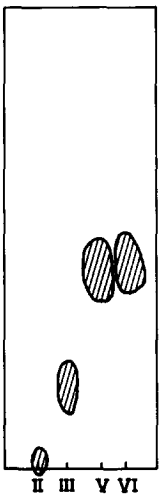

FIGURE IV. Thin layer chromatography (silica gel, ethyl acetate—acetone 1:1) of compounds II, III, V, and VI.

will convert $\underline{N}^6$-methyladenosine to inosine at a rate ~625 times lower than with the natural substrate, whereas $\underline{N}^6$,$\underline{N}^6$-dimethyladenosine was bound to the enzyme but did not undergo deamination. The present examination of III and V demonstrates that the $\underline{N}^6$-methyl derivative III is slowly deaminated to the hypoxanthine product whereas V remains unchanged after 64 hours (see Materials and Methods). The observed loss of radioactivity from the biologically methylated base (4), which was attributed to deaminase activity associated with nucleoside phosphorylase, would indicate the $\underline{N}^6$-monomethyl isomer (III) if enzyme specificities are equivalent.

The present study makes the $\underline{N}^1$ (II), $\underline{N}^6$ (III), and $\underline{N}^6$,$\underline{N}^6$-di (V) methyl derivatives of $\underline{O}^{2'}$-methyladenosine (I) readily available for direct comparison with biologically derived samples. Tlc systems which easily and cleanly resolve III and V, and a simple enzyme assay which differentiates III and V (both applicable on "tracer-level" scales) have been devised. These results provide means for the defined evaluation of the methylated adenosine nucleosides in mRNA "caps".

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