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5-Methoxyuridine : a new minor constituent located in the first position of the anticodon of $tRNA^{Ala}$, $tRNA^{Thr}$, and $tRNA^{Val}$ from Bacillus subtilis

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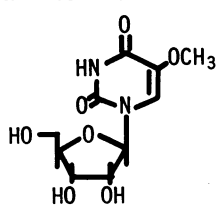
ABSTRACT

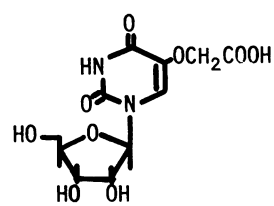
The sequences of the anticodon of $tRNA^{Ala}$, $tRNA^{Thr}$, and $tRNA^{Val}$ from *Bacillus subtilis* W 168 were N-G-C, N-G-U, and N-A-C, respectively. A new minor constituent, N, occupied the first position of the anticodon of each tRNA. N was identified as 5-methoxyuridine (mo^5U, Figure 1) by comparison of its UV absorption spectra, Rf values in thin-layer chromatography using several solvent systems and mass spectra with those of chemically synthesized specimen.

INTRODUCTION

We have been interested in the structure and the function of minor constituents in tRNA. The first position of the anticodon of tRNA recognizing three codons is generally occupied by modified nucleoside such as uridin-5-oxyacetic acid[1] (V, Figure 1) or inosine[2] in case of *E. coli* tRNAs. In the course of structural studies on *B. subtilis* $tRNA^{Ala}$, $tRNA^{Thr}$, and $tRNA^{Val}$, we found that each of three tRNAs has an unidentified minor component in the first position of the anticodon. These three minor components were found to be identical from their UV spectra and two-dimentional thin-layer chromatographic mobilities. Here, we describe the isolation of this nucleoside N and its identification as mo^5U.



(I)



(II)

Figure 1 Structures of (I) mo^5U and (II) V.

Abbreviations. mo^5U : 5-methoxyuridine, V : uridin-5-oxyacetic acid, m^6A : 6-methyladenosine, t^6A : *N*-[(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]threonine, TMS : trimethylsilyl, X-$(TMS)_3$: tris-trimethylsilyl nucleoside

MATERIALS AND METHODS

Materials

Crude tRNA from *B. subtilis* was prepared as described previously[3]. $tRNA^{Ala}$, $tRNA^{Thr}$, and $tRNA^{Val}$ were purified by combined use of DEAE-Sephadex A-50[4], Sepharose 4B[5], and benzoylated DEAE-cellulose[6] column chromatographic systems. These three tRNAs were over 90% pure with respect to amino acid acceptor activity and the chromatographic pattern of RNase T_1 or RNase A digest of tRNA. Details of these purification procedures will be published elsewhere. RNase T_1, T_2, and U_2 were purchased from Sankyo Co. Ltd. *E. coli* alkaline phosphatase was obtained from Boehlinger Mannheim Yamanouchi Co. Silkworm endonuclease was a gift from Dr. J. Mukai of Kyushu University. Nuclease P_1 from *Penicillium citrinum* was a product of Yamasa Shoyu Co. Ltd. DEAE-Sephadex A-25 was a product of Pharmacia Fine Chemicals. DEAE-cellulose was obtained from Brown Co. Avicel SF cellulose plate and Whatman 3MM paper were purchased from Funakoshi Pharmaceutical Co. Toyo-roshi No. 51A was obtained from Toyo-roshi Co. Ltd. Trimethylsilylating reagent [*N,O*-Bis(trimethylsilyl)trifluoroacetamide containing 1% trimethyl chlorosilane] was kindly provided by Dr. S. Nishimura of National Cancer Center Research Institute.

Paper and thin-layer chromatography

All of chromatographies were carried out by ascending technique using following solvent systems : Solvent A, 1-butanol-H_2O (84:16), Solvent B, *tert*-butanol-ammonium formate, pH 3.5 (1:1)[7], Solvent C, ethanol-1 M ammonium acetate, pH 7.5 (7:3), Solvent D, isobutyric acid-0.5 N NH_4OH (5:3), Solvent E, 2-propanol-concentrated HCl-H_2O (70:15:15), Solvent F, 1-propanol-concentrated NH_4OH-H_2O (55:10:35). Two-dimentional thin-layer chromatography for base analysis was performed as described by F. Kimura-Harada et al.[8] using Avicel SF cellulose plate with solvent D (first dimention) and solvent E (second dimention). Spots were detected on chromatograms under ultraviolet lamp at 253.7 nm.

Paper electrophoresis

Paper electrophoresis was carried out on Toyo-roshi No. 51A paper at 30 V/cm with 0.05 M triethylammonium bicarbonate buffer (pH 7.5) for 30 min.

Enzymatic digestion

Excepting for described below, enzymatic digestion was carried out as reported by F. Harada et al.[9] For nuclease P_1 digestion, 2 OD_{260} units of oligonucleotide and 0.5 unit of the enzyme were incubated in 0.01 ml of water at 37° for 1 hr. For silkworm endonuclease digestion, 5 OD_{260} units

of oligonucleotide and 6 units of the enzyme were dissolved in 0.02 ml of 0.05 M sodium carbonate buffer (pH 10.5) containing 0.1 M sodium chloride and 0.5 mM magnesium acetate, and incubated at 37° for 5 hr.

DEAE-Sephadex A-25 chromatography at neutral and acid conditions

Solid urea was added to the solution of RNase digest to a concentration of 7 M. The mixture was adsorbed on a column of DEAE-Sephadex A-25 (0.5 x 150 cm). Elution was carried out with a sodium chloride linear gradient from 0.14 to 0.60 M (500 ml x 2) in the presence of 0.02 M Tris-HCl (pH 7.5) and 7 M urea. The fractions of an appropriate peak were pooled and diluted 5-fold with water and applied to a column of DEAE-cellulose (1 x 5 cm). The column was washed with small volume of water and connected to the top of a column of DEAE-Sephadex A-25 (0.5 x 100 cm) equilibrated with 0.06 N HCl containing 7 M urea. Elution was carried out with a sodium chloride linear gradient from 0 to 0.40 M (250 ml x 2) in the presence of 0.06 N HCl and 7 M urea. The relevant pooled fractions were finally desalted by the method reported previously[9].

Mass spectrometry of trimethylsilyl derivatives

Conversion to the volatile trimethylsilyl (TMS) derivatives was performed by the method described by F. Kimura-Harada et al.[8] Approximately 0.5 OD_{280} unit of a natural or synthetic nucleoside was modified with an excess of *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethyl chlorosilane. The mass spectra of TMS derivatives were recorded on a JEOL JMS-01SG-2 mass spectrometer with ionizing electron energy of 75 eV using direct inlet probe.

Chemical synthesis of 5-methoxyuridine

5-Hydroxyuridine was synthesized by the method described by T. Ueda[10]. 5-Hydroxyuridine (30 mg) was further methylated by 20 µl of dimethyl sulfate in 0.6 ml of 0.1 N NaOH. A reaction mixture was chromatographed on Whatman 3MM paper using solvent D. The band containing mo^5U was extracted with water and the extract was further purified by paper chromatography in solvent E. These procedures gave a 25% yield of mo^5U. In addition to mo^5U and the raw material, the methylated products contained 5% of 5-hydroxy-3-methyluridine and 4% of 5-methoxy-3-methyluridine.

RESULTS

The nucleotide sequences of the oligonucleotides containing N

$tRNA^{Ala}$ (200 OD_{260} units) was hydrolyzed with RNase T_1 and fractionated on a column of DEAE-Sephadex A-25. The oligonucleotide containing N

was eluted as a pentanucleotide (Figure 2a, peak 5). It was digested with silkworm endonuclease, which cleaves a pentanucleotide to two halves bearing 5'-phosphate or 3'-hydroxyl group[11], and the products were separated by paper electrophoresis. Two main spots were observed. The faster moving spot was further treated with RNase T_2 and the digest was chromatographed two-dimentionally on thin-layer plate. The separated three spots were identified as pUp, Np, and Gp, respectively. Therefore, this fragment was identified as pU-N-Gp. The slower one was also treated with RNase T_2 and was subjected to base analysis. Two spots were observed and identified as Cp and uridine. Thus the second fragment was found to be C-U. From these results, the nucleotide sequence of the pentanucleotide containing N from $tRNA^{Ala}$ was determined as C-U-U-N-Gp.

$tRNA^{Thr}$ (107 OD_{260} units) was hydrolyzed with RNase T_1 and the oligonucleotide containing N was given as a pentanucleotide (Figure 2b, peak 5). In the same manner as described above, the pentanucleotide containing N was split with silkworm endonuclease mainly to A-C and pU-N-Gp. As a result,

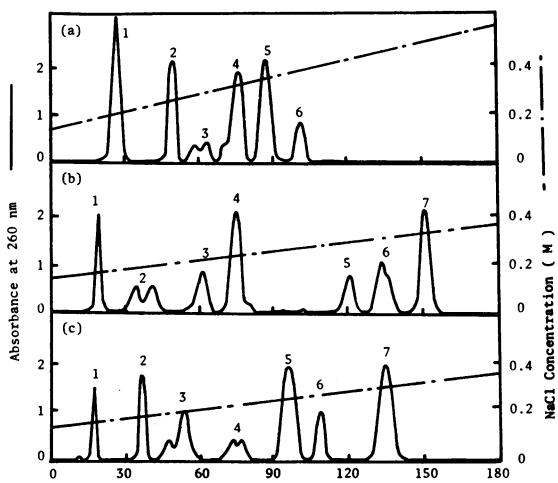


Figure 2 DEAE-Sephadex A-25 column chromatography at pH 7.5.
(a) RNase T_1 digest of $tRNA^{Ala}$ (200 OD_{260} units) was chromatographed and fractions of 4.5 ml of effluent were collected. Peak 5 was further divided into three components on a column of acid DEAE-Sephadex A-25, and the last eluting peak contained N.
(b) RNase T_1 digest of $tRNA^{Thr}$ (107 OD_{260} units) was chromatographed and 2.0 ml-fractions were collected. Peak 5 was composed of a single oligonucleotide containing N.
(c) RNase T_1 digest of $tRNA^{Val}$ (100 OD_{260} units) was chromatographed and fractions of 3.0 ml of effluent were collected. Peak 7 was further separated into two components by acid DEAE-Sephadex A-25 chromatography. The last eluting peak was nonanucleotide containing N.

its sequence was determined as A-C-U-N-Gp.

As shown in Figure 2c, peak 7, RNase T_1 digest of $tRNA^{Val}$ (100 OD_{260} units) gave a nonanucleotide containing N, which was partially digested with RNase U_2. The resulting oligonucleotides were separated by two-dimentional paper chromatography using solvent D and solvent B. The first of three spots observed was further analyzed by the same method as the pentanucleotides from $tRNA^{Ala}$ and $tRNA^{Thr}$, and its sequence was determined as C-C-U-N-Ap. Second spot was found to be C-m^6A-Ap by analysis of nuclease P_1 digest. The third spot was identified as Gp from its spectrum and thin-layer chromatographic behavior. Meanwhile, one of the digestion products of the nonanucleotide with RNase A was m^6A-A-Gp. In all respects, the nucleotide sequence of the nonanucleotide from $tRNA^{Val}$ was unambiguously determined as C-C-U-N-A-C-m^6A-A-Gp.

Total primary sequence analysis of each tRNA was carried out by the conventional methods such as complete or partial digestion with RNase T_1 or RNase A (details of these analyses will be reported elsewhere). These three oligonucleotides were located in the anticodon loop of $tRNA^{Ala}$, $tRNA^{Thr}$, and $tRNA^{Val}$, respectively. In particular, N was occupied the first position of the anticodon of each tRNA. The anticodon structures of three tRNAs are shown in Figure 3.

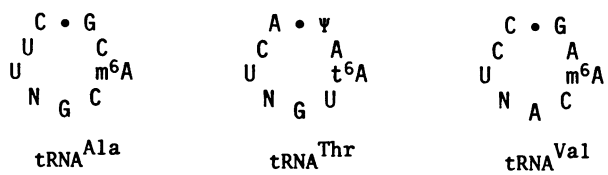


Figure 3 Structures of the anticodon loop of $tRNA^{Ala}$, $tRNA^{Thr}$, and $tRNA^{Val}$ from B. subtilis.

Identification of N as 5-methoxyuridine

In order to characterize the structure of N, the isolation procedure was performed on large scale. $tRNA^{Ala}$ (1000 OD_{260} units) was hydrolyzed with 2500 units of RNase T_1 in 5 ml of 0.05 M Tris-HCl (pH 7.5) at 37° for 16 hr. The RNase T_1 digest was fractionated on a column of DEAE-Sephadex A-25 (1 x 100 cm) using a sodium chloride linear gradient from 0.14 to 0.60 M (3 l x 2) in the presence of 0.02 M Tris-HCl (pH 7.5) and 7 M urea. The pentanucleotide fractions were further chromatographed at acid pH on a column of DEAE-Sephadex A-25. The peak containing N was desalted and the nucleotide Np was isolated from this pentanucleotide by silkworm endonuclease digestion and paper chromatography as described above. Np was

hydrolyzed with *E. coli* alkaline phosphatase and the resulting nucleoside N was purified by paper chromatography in solvent D. The yield of N was approximately 10 OD_{280} units.

From its UV spectra ($\lambda_{max}^{pH\ 2}$ 279 nm, $\lambda_{max}^{pH\ 12}$ 277 nm) (Figure 4, a, b, and

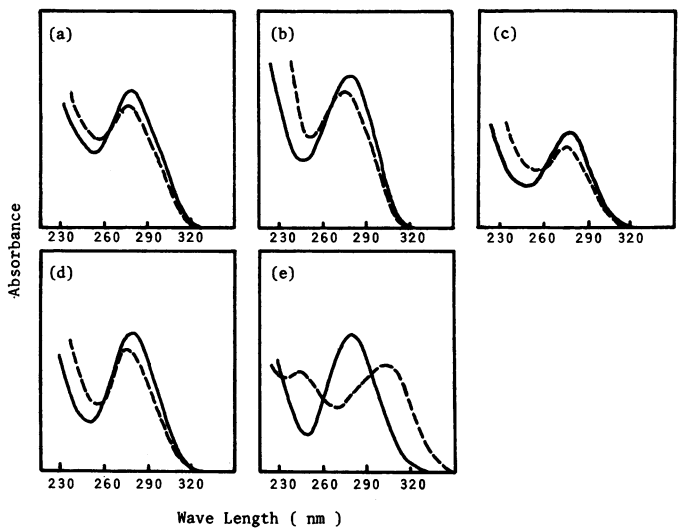


Figure 4 UV absorption spectra of natural N from (a) $tRNA^{Ala}$, (b) $tRNA^{Thr}$, and (c) $tRNA^{Val}$, and chemically synthesized (d) mo^5U and (e) 5-hydroxy-3-methyluridine. pH 2, ———; pH 12, - - - - - - -.

c), two-dimentional thin-layer chromatographic behavior (N moves at the same position as U), and from the fact that the phosphodiester bond of N was hydrolyzed by RNase A not by RNase T_1, it was concluded that N is a derivative of uridine. Compairing the UV absorption spectra of N with those of several 5-substituted uridine derivatives such as uridin-5-oxyacetic acid ($\lambda_{max}^{pH\ 2}$ 276 nm, $\lambda_{max}^{pH\ 12}$ 275 nm)[1], 5-methoxyuridine ($\lambda_{max}^{pH\ 2}$ 279 nm, $\lambda_{max}^{pH\ 12}$ 277 nm)[12], and 5-hydroxyuridine ($\lambda_{max}^{pH\ 2}$ 280 nm, $\lambda_{max}^{pH\ 12}$ 306 nm)[10], it was suggested that the oxy linkage (-O-) is attached to the 5'-position of the uracil ring.

As attempts to directly record the mass spectrum of free nucleoside N were unsuccessful, N was modified to the more volatile trimethylsilyl derivative. As shown in Figure 5a, mass spectral analysis of N-$(TMS)_3$ indicated the molecular ion peak at m/e 490. Compaired with that of U-$(TMS)_3$ (m/e 460), N-$(TMS)_3$ is 30 mass units higher. This excess mass units correspond to $-CH_2OH$, $-OCH_3$, or -OH plus $-CH_3$. Namely, possible structure of N is regarded as 5-hydroxymethyluridine, 5-methoxyuridine, or 5-hydroxy-3-methyluridine. However, 5-hydroxymethyluridine was excluded on account of the

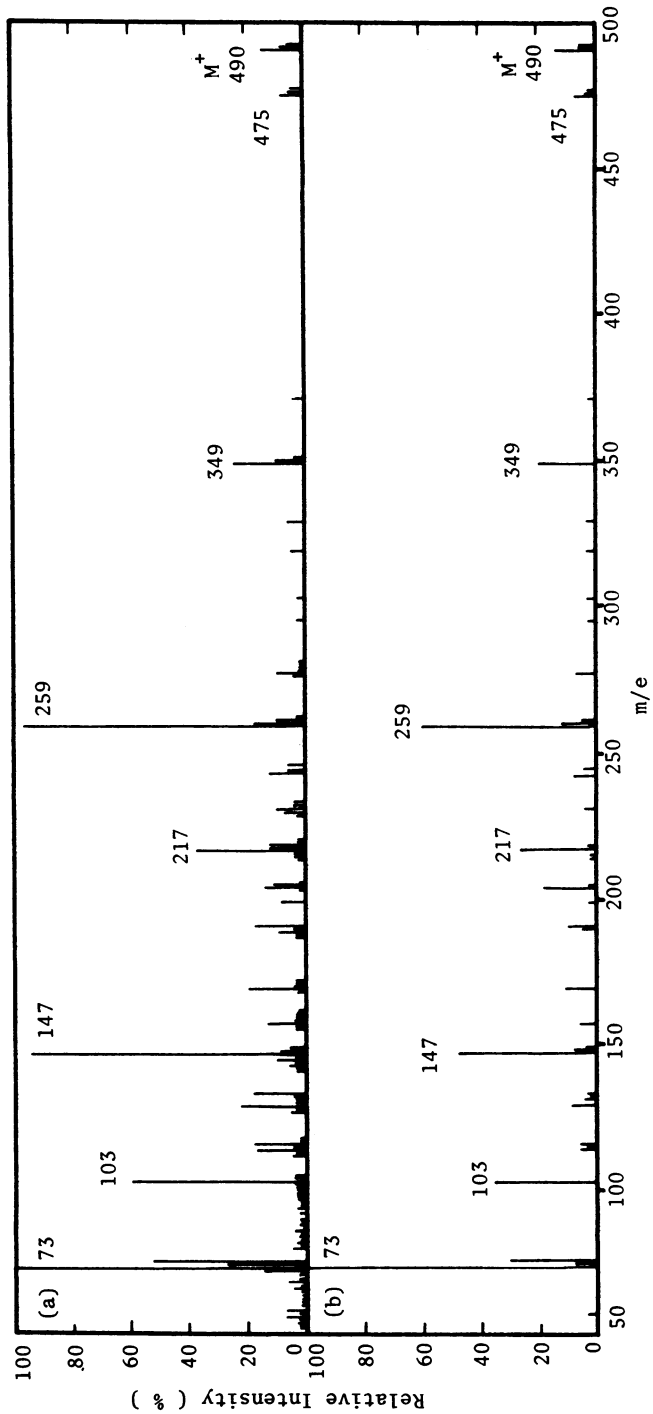


Figure 5 Mass spectra of trimethylsilylation products of (a) natural and (b) synthetic mo^{5}U.

difference of UV absorption spectra ($\lambda_{max}^{pH\ 2}$ 264 nm, $\lambda_{max}^{pH\ 12}$ 263 nm)[13] and 5-hydroxy-3-methyluridine was also excluded because of the fact that 3-substituted uridine derivatives are resistant to the action of RNase A[14]. Thus the most probable structure of N is considered to be mo^5U. In order to obtain direct proof, mo^5U was synthesized chemically.

Figure 4 shows a comparison of UV spectra of N from three tRNAs with those of synthetic samples. The spectra of N from each tRNA are identical with those of mo^5U at different pH values. Rf values of N, U, and methylated uridine derivatives in thin-layer chromatography are shown in Table 1.

Table 1. Rf values of N and related compounds in several solvent systems.

Compound \ Solvent system	A	B	C	D	E	F
Nucleoside N	0.19	0.70	0.62	0.50	0.65	0.75
Uridine	0.19	0.66	0.61	0.47	0.63	0.74
5-Methoxyuridine	0.19	0.70	0.62	0.50	0.65	0.75
5-Hydroxy-3-methyluridine	0.28	0.78	0.64	0.59	0.83	0.71

These data clearly demonstrate that N is mo^5U since N behaved identically with the synthetic specimen in all respects. The identity of N with mo^5U was confirmed by mass spectra of these two compounds trimethylsilylated. Both N-$(TMS)_3$ and mo^5U-$(TMS)_3$ gave a molecular ion peak at m/e 490. Moreover, the fragmentation patterns of two derivatives are almost identical as shown in Figure 5. On the other hand, 5-hydroxy-3-methyluridine was trimethylsilylated at four positions in the same reaction condition, giving a larger molecular ion : m/e 562 (data are not shown). Thus mass spectral analysis also gave reliable evidence for the identity of N with mo^5U.

DISCUSSION

We could not detect mo^5Up in RNase T_2 hydrolyzate of whole tRNA molecule, because mo^5Up was chromatographed at the same position as Up on a thin-layer plate for base analysis. The separation of mo^5U from U is difficult in paper or thin-layer chromatography with usual solvent systems. In case of RNase T_2 hydrolyzate of C-U-U-mo^5U-Gp from $tRNA^{Ala}$, we found the maximum UV absorption, at pH 2, of a spot corresponding to Up not at 262 nm but at 265 nm. Therefore, we suspected the presence of a new modified nucleotide. Digestion of this oligonucleotide with silkworm endonuclease resulted two fragments, i.e. C-U and pU-mo^5U-Gp. RNase T_2 digest of the latter fragment gave pUp, mo^5Up, and Gp, then U and mo^5U can be separated easily as a nucleoside diphosphate and a nucleoside monophosphate, respec-

tively. A sharp distinction between mo^5Up and Up was established by measurement of UV absorption spectra. If these analyses were executed with [^{32}P]-labeled tRNA samples using radioactivity as a sole detective device, mo^5U would be identified as Up and never be detected. Since the nucleoside next to the 5'-end of the anticodon is usually U, and mo^5U is located the first position of the anticodon, mo^5U will be contained in an RNase T_1-fragment almost always together with U. These two components can be separated in chromatography with usual solvent systems only when they hold different number of phosphate, namely one is nucleoside and the other is nucleotide, or one is nucleoside monophosphate and the other is nucleoside diphosphate.

This is the first evidence of the presence of mo^5U in tRNA. We were recently informed that M. Albani and H. Kersten also detected mo^5U independently of our work as a methylated uridine derivative in tRNAs of *B. subtilis* and several gram-positive microorganisms (personal communication from Dr. H. Kersten). Our present work clearly demonstrates the existence of mo^5U in the particular tRNAs from *B. subtilis*, and, moreover, in the specific site of tRNA molecule, i.e. in the first position of the anticodon. This fact strongly suggests that mo^5U plays an important role in codon-anticodon base pairing. V in the wobble position of the anticodon of *E. coli* tRNAs, of which structure is analogous to mo^5U (see Figure 1), is recognized by A and G, and fairly well by U in the third letter of codons when tested in tRNA-ribosome binding experiments[15]. Modification at the 5'-position of the uracil ring might promote this tendency. Studies of the recognition of mo^5U is under progress.

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