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3-(3-Amino-3-carboxypropyl)uridine: a Novel Modified Nucleoside Isolated from *Escherichia coli* Phenylalanine Transfer Ribonucleic Acid†

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ABSTRACT: An unknown modified nucleoside located in the extra-region of *Escherichia coli* $tRNA^{Phe}$ has been characterized as 3-(3-amino-3-carboxypropyl)uridine (4abu^{3}U). Its identity was established unambiguously by its ultraviolet absorption spectrum, thin-layer chromatographic and electrophoretic mobilities, chemical reactivity, mass spectra, and nuclear magnetic resonance spectrum, and by comparison with those of a chemically synthesized authentic sample.

The primary sequence of *Escherichia coli* $tRNA^{Phe}$ was previously reported by Barrell and Sanger (1969). It was shown that an unknown modified component designated as X was located in the extra-region. The exact chemical structure of X, hereafter designated as N*,[1] has not been determined. We observed that phenylalanine acceptor activity of *E. coli* $tRNA^{Phe}$ was extensively inactivated by chemical modification with cyanogen bromide, suggesting that a component other than 4-thiouridine, possibly N*, had reacted with cyanogen bromide (Saneyoshi and Nishimura, 1971). It was necessary to determine the structure of N* in order to understand its chemical reactivity, function, and biosynthesis.

We present evidence that N* is 3-(3-amino-3-carboxypropyl)-uridine (4abu^{3}U) (I). In order to characterize the structure of N*, the nucleoside was isolated in large scale from purified *E. coli* $tRNA^{Phe}$, and its properties were thoroughly examined. For final characterization, 4abu^{3}U was chemically synthe-

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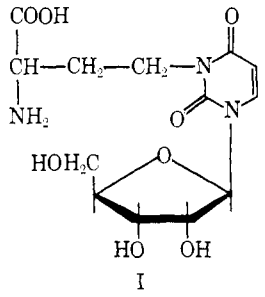
[1] Abbreviations used are: 4abu^{3}U, 3-(3-amino-3-carboxypropyl)-uridine; N*, unidentified modificd nucleoside located in the extra-region of *E. coli* $tRNA^{Phe}$ and characterized as 4abu^{3}U; N**, a derivative of 4abu^{3}U found in small amount in *E. coli* $tRNA^{Phe}$; m^{7}G, 7-methylguanosine; s^{4}U, 4-thiouridine; Bz, benzoyl group; A_{260} unit, the amount of material giving an absorbance of 1.0 at 260 nm when dissolved in 1 ml of water and measured in a cell of 1-cm light path; M, molecular ion.

sized. It has been shown that the synthetic material is identical with N* with respect to ultraviolet absorption spectra, thin-layer chromatographic mobilities, and mass spectrum.



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Materials and Methods

Isolation of E. coli tRNA^{Phe}. Unfractionated tRNA was prepared from *E. coli* B cells harvested in the late-log phase of growth as described by Zubay (1962), except that treatment with alkali was omitted. *E. coli* tRNA thus obtained was first fractionated by DEAE-Sephadex A-50 column chromatography at pH 7.5 (Nishimura, 1971) and at pH 4.0 (Yoshida *et al.*, 1971). Final purification of tRNA^{Phe} was achieved by the reverse-phase partition column chromatography (RPC-1) (Kelmers *et al.*, 1965; Nishimura, 1971).

*Isolation of N*p and N*. E. coli* tRNA^{Phe} (800 A_{260} units) was hydrolyzed by 1.6 mg of RNase A in 1 ml of 0.05 M triethylammonium bicarbonate buffer (pH 7.8) at 37° for 18 hr. The hydrolysate was applied to DEAE-cellulose paper (Whatman DE82) as a streak 40 cm wide, and fractionated by electrophoresis at pH 1.9 with 800 V for 6 hr according to the procedure described by Barrell and Sanger (1969). The ultraviolet absorbing band corresponding to m^{7}G-N*-Cp was eluted with 2 M triethylammonium bicarbonate (pH 7.8), evaporated to dryness, and dissolved in 1 ml of water. The solution containing m^{7}G-N*-Cp was incubated with 10 units of RNase T_2 as described previously (Harada *et al.*, 1971). The digest was applied to two Whatman 3MM papers, and fractionated by two-dimensional paper chromatography as described previously (Harada and Nishimura, 1972). N*p was located in the position where Gp is normally found (see Figure 1). Since the digest was not contaminated with Gp, N*p was isolated as a single component by this procedure. The spot containing N*p was eluted with water to yield approximately 5 A_{260} units of material. Nucleoside N* was obtained from N*p by treatment with *E. coli* alkaline phosphomonoesterase as previously described (Harada *et al.*, 1971). It should be noted that a compound similar to N*p was also found in a position close to N*p in the two-dimensional chromatogram (see Figure 1). The yield of this compound, N**p, was approximately one-fifth that of N*p. Due to an insufficient amount of N**, structural characterization was carried out only on the major component, N*.

Mass Spectrometry of Trimethylsilyl Derivative. Conversion of N* to the trimethylsilyl derivative (McCloskey *et al.*, 1968a) was carried out as described previously (Kimura-Harada *et al.*, 1972). Mass spectra were recorded by using an LKB 9000 instrument, with ionizing electron energy of 70 eV, and ion source temperature 250°. The sample was introduced by direct probe after removal of silylation reagents under vacuum. The quantity of nucleoside used for each experiment was 0.2–0.4 A_{260} unit.

Proton Nuclear Magnetic Resonance (nmr) Spectroscopy. The nmr spectrum of N*p was obtained with a JEOL 100 MHz FT spectrometer (PS100/PFT-100), with 18000 times

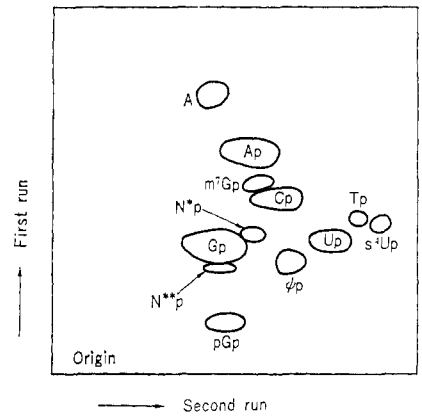


FIGURE 1: Schematic diagram showing the position of the modified nucleosides, N*p and N**p, in two-dimensional thin-layer chromatography.

accumulation, using 3 A_{260} units of the nucleotide dissolved in 0.2 ml of D_2O. Exchangeable protons of N*p were replaced with deuterium prior to measurement of the spectrum. This treatment was repeated five times with 99.9% D_2O.

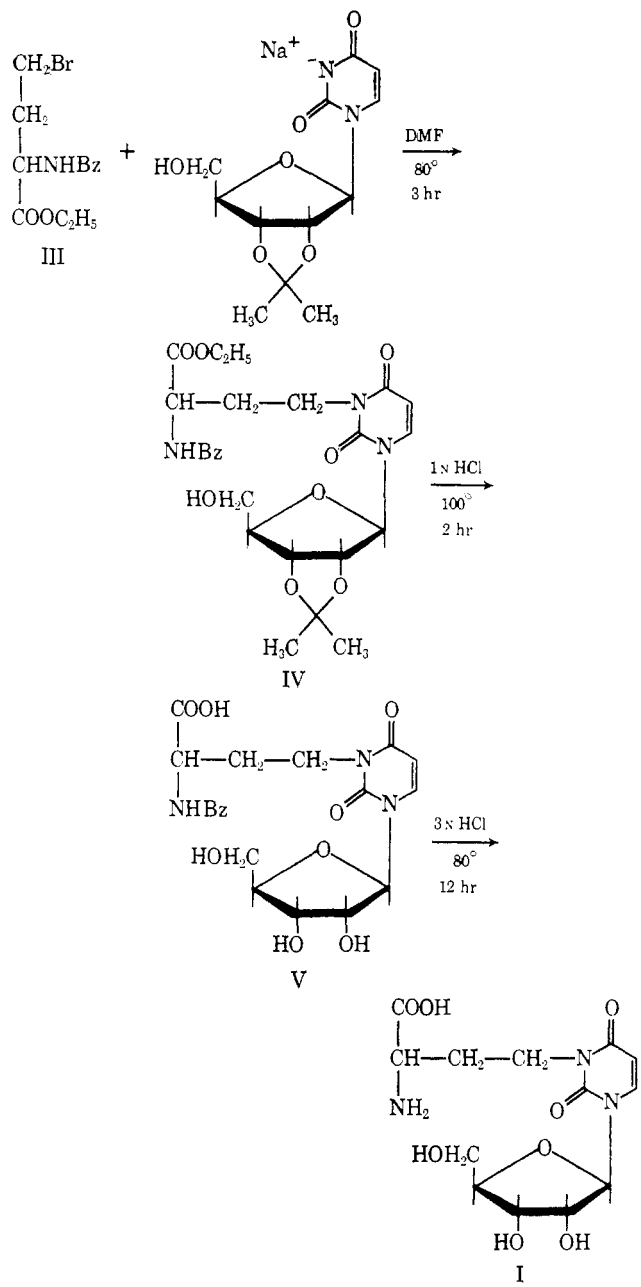
Solvent Systems for Paper and Thin-Layer Chromatography. The solvent systems used for two-dimensional paper chromatography were: first dimension, solvent 1 [isobutyric acid–0.5 M NH_4OH (5:3, v/v)]; second dimension, solvent 2 [2-propanol–concentrated HCl–water (70:15:15, v/v)]. Other solvent systems used for characterization of N* were: solvent 3, 1-butanol–acetic acid–water (4:1:2, v/v); solvent 4, 2-propanol–concentrated NH_4OH–water (7:1:2, v/v); solvent 5, ethyl acetate–1-propanol–water (4:1:2, v/v).

Materials. Pancreatic RNase (RNase A) (five-times recrystallized), RNase T_2, and *E. coli* alkaline phosphomonoesterase were obtained from Sigma Chemical Co., Sankyo Co. Ltd., Tokyo, and Miles Laboratories, Inc., respectively. *N,O*-Bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane used for trimethylsilylation of N* was purchased from Regis Chemical Co., Chicago, Ill. Deuterated reagents, *i.e.*, *N,O*-bis(trimethylsilyl)trifluoroacetamide-d_{18} and trimethylchlorosilane-d_9, were purchased from Merck Sharp & Dohme of Canada, Pointe Claire, Quebec. Whatman No. 3MM and DE82 papers were the products of W & R Balston Ltd. Thin-layer glass plates coated with Avicel SF cellulose were purchased from Funakoshi Pharmaceutical Co., Tokyo. D_2O of 99.9% purity was purchased from Merck.

Chemical Synthesis of 4abu^{3}U. L-α-Benzamido-γ-butyrolactone (II) was synthesized from 1.19 g of L-α-amino-γ-hydroxybutyric acid by the procedure described by Knobler and Frankel (1958), and Frankel and Knobler (1958). The product was recrystallized from water to afford 1.5 g of II in 73% yield, mp 138–142° [lit. 141–142°, DL mixture (Knobler and Frankel, 1958)]. Then the compound II was converted to ethyl L-α-benzamido-γ-bromobutyrate (III) by reacting with dried hydrogen bromide according to the procedure described by Knobler and Frankel (1958), and Frankel and Knobler (1958). The product was crystallized from ethyl alcohol. The yield was 78%, mp 95–96° [lit. 83–84°, DL mixture (Knobler and Frankel, 1958)].

Compound III (444 mg) dissolved in 1 ml of dimethylformamide was added to 8 ml of dimethylformamide containing 200 mg of 2′,3′-*O*-isopropylideneuridine. The mixture was kept at 80–85°, and the reaction was monitored by analyzing the reaction mixture by silica gel G thin-layer chromatography. The thin-layer chromatogram developed with a solvent system of chloroform–methanol (19:1 v/v) showed three

SCHEME I: Route of Chemical Synthesis of 4abu^{3}U.



spots. One of the spots near the front corresponded to the activated amino acid and another spot near the origin corresponded to isopropylideneuridine. A new spot appeared in a middle position between the two spots during the course of the reaction, and its amount increased with time of incubation. After 3 hr of incubation, the reaction mixture was evaporated to dryness *in vacuo*. The residue was subjected to preparative silica gel G thin-layer chromatography. The middle band on the plate was eluted with chloroform–methanol (4:1, v/v), and the eluate was evaporated to dryness under reduced pressure. The hydrolysis of IV to remove 2′,3′-*O*-isopropylidene and ethyl ester groups was carried out by heating in aqueous 1 N HCl at 100° for 3 hr. The product thus obtained (V) was crystallized from an ethanol–ether mixture. Yield was 55% (200 mg). The benzoyl group of V was removed by treatment with aqueous 3 N HCl at 80° for 12 hr. The hydrolysate was applied to a column of Dowex 50 (H*), and washed with water to elute unhydrolyzed compound V. Then the product I was eluted with aqueous 1 N HCl. The eluate was evaporated to

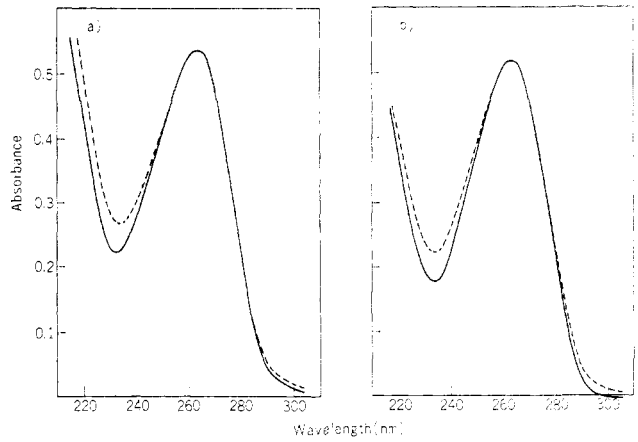


FIGURE 2: Ultraviolet absorption spectra of (a) N* and (b) authentic 4abu^{3}U: (—) pH 6 and 2; (- - - - - -) pH 12.

dryness *in vacuo* to afford 120 mg of I in crystalline form. The route of chemical synthesis of 4abu^{3}U was illustrated in Scheme I. The compound I (4abu^{3}U) exhibited λ_{max} (pH 1, 7.2 and 13) 263 nm; ϵ (pH 1, 7.2, and 13), 8.5×10^3; mp 161–163°. *Anal.* Calcd for $C_{13}H_{19}N_3O_8 \cdot HCl \cdot H_2O$: C, 39.05; H, 5.54, N, 10.51. Found: C, 39.23, H, 5.15; N, 10.17.

Results

*General Properties of N**. It was previously suggested that N* is a derivative of uridine (Barrell and Sanger, 1969; Yarus and Barrell, 1971). Figure 1 shows position of N*p in the two-dimensional thin-layer chromatogram. N*p migrated relatively little in both directions, unlike Up, suggesting that N* contains a hydrophilic side chain. Figure 2 shows ultraviolet absorption spectra of N* at different pH values. λ_{max} of N* in both alkaline and acidic conditions was found to be 263 nm, and its molecular extinction coefficient was not altered by changing the pH. The spectrum is similar to that of 3-methyluridine (Hall, 1971), suggesting that N* is a derivative having an alkyl substituent at position N-3 of uridine. It was known that the phosphodiester bond of N* is resistant to attack by RNase A (Barrell and Sanger, 1969), which also suggested that N* is an N-3-substituted uridine derivative.

It is noteworthy to mention that N* was found to react with ninhydrin; 0.1 A_{260} unit of N* was spotted on a Avicel SF cellulose thin layer glass plate. The plate was sprayed with 1-butanol saturated with water containing 0.1% ninhydrin, and heated at 80° for 5 min. N* gave a reddish-brown color. The sensitivity of the reaction of N* with ninhydrin was comparable to that of an α-amino acid, suggesting that N* contains an α-amino-α-carboxy group. Table I shows electrophoretic mobilities of N* and acetic anhydride treated N* as compared with several standard nucleosides and nucleotides. Under neutral conditions, both N* and acetic anhydride treated N* (possibly containing an acetylated α-amino group) migrated to the anode, suggesting that N* possesses a free carboxyl group.

*Mass Spectrometry of the Trimethylsilyl Derivative of N**. Attempts to record the mass spectrum of free N* were unsuccessful because of its high polarity. Therefore conversion to the more volatile trimethylsilyl derivative was carried out. The mass spectrum of the trimethylsilylation product is shown in Figure 3. The spectrum exhibits a molecular ion peak of *m/e* 633, the identity of which is confirmed by *m/e* 618 due to the characteristic loss of a trimethylsilyl methyl group (McCloskey *et al.*, 1968). The spectrum of the corresponding

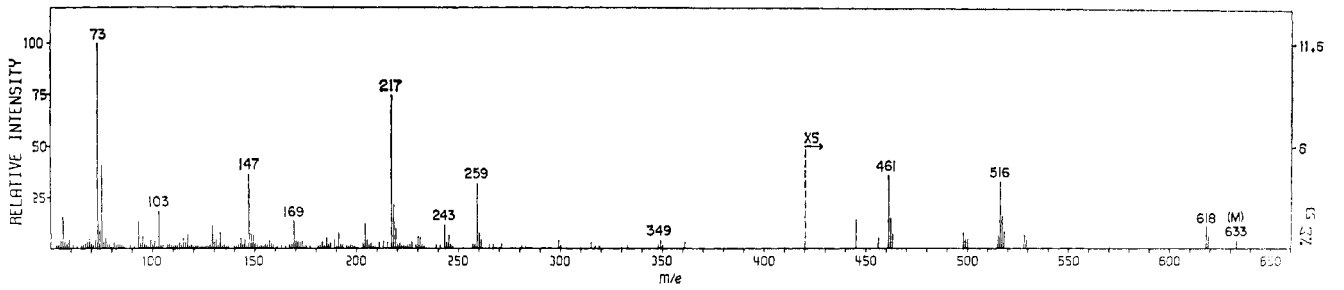
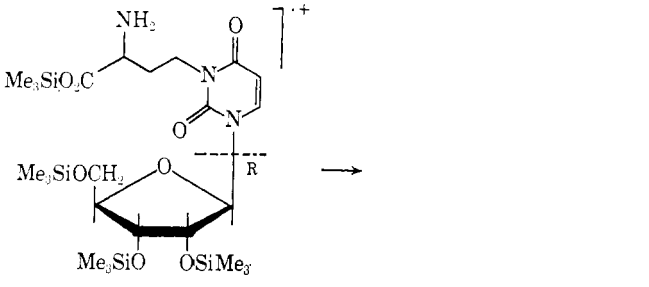


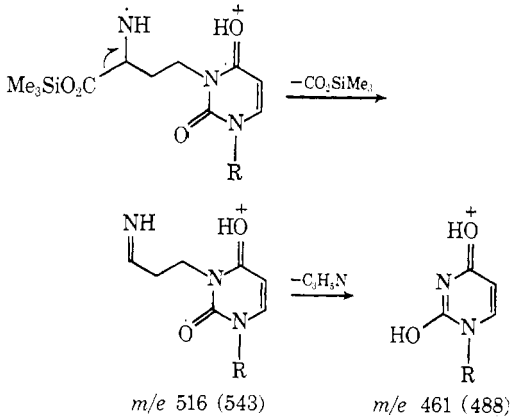
FIGURE 3: Mass spectrum of the trimethylsilylation product of N*.

silyl-d_9 derivative showed a molecular ion shift of 36 mass units, thereby indicating the presence of four silyl groups (McCloskey *et al.*, 1968), which established the molecular weight of free N* as 345. In some derivative preparations a higher silyl homolog of molecular weight 705 was observed, the spectrum of which showed the additional silyl group to be in the side chain. Numerous ions in the low mass region of the spectrum shown in Figure 3 are common to all ribonucleosides having an unmodified sugar: *m/e* 349, 259, 243, 217, 169, 147, and 103 (McCloskey *et al.*, 1968a). The abundant ions of mass 73 (Me_3Si^+) and 75 (Me_2SiOH^+) are usually produced by trimethylsilyl ethers and bear no structural information.

The prominent fragment ions of mass 461 and 516 do not correspond to common nucleoside fragmentation reactions and are therefore associated with modification in the base. Initial loss of 117 mass units from the molecular ion to produce *m/e* 516 is followed by expulsion of 55 mass units as marked by a metastable peak at *m/e* 412. The reactions are consistent with the structures and fragmentation reactions shown below. Mass values in parentheses refer to the analogous ions from the mass spectrum of the trimethylsilyl-d_9 derivative. Since the side chain is known to contain carboxyl and amino groups, its composition in the derivative can be reasonably postulated as $C_4H_6NO_2SiMe_3$. Initial transfer of a



m/e 633 (669)



m/e 516 (543) *m/e* 461 (488)

labile hydrogen to the base has analogy in the common nucleoside fragmentation reactions (Biemann and McCloskey, 1962; Shaw *et al.*, 1970) and can readily induce loss of the silyl ester radical. Loss of the 55 mass unit neutral species is rationalized in terms of a hydrogen transfer reaction with breakage of the side chain–N-3 bond. The latter reaction is reminiscent of glycosidic bond cleavage during formation of the common "base + H" ion (McCloskey, 1974) which is not observed when the glycosidic bond is linked to carbon rather than nitrogen (Townsend and Robins, 1969; Hecht *et al.*, 1969; Rice and Dudek, 1969). The 516 → 461 transition therefore strongly supports N-3 as the site of side chain attachment.

*Nmr Spectroscopy of N**. In order to determine whether the side chain of N* contains $-CH_2CH_2-$ or $-CH(CH_3)$ groups, the nmr spectrum of N*p was obtained by the procedure described in Materials and Methods. The nmr spectrum of N*p as shown in Figure 4 indicated the presence of protons at the C-5 and C-6 positions of the uridine skeleton and an anomeric proton (C-1′) located in the ordinary range. In addition to these signals, a multiplet at 2.1 ppm was observed, which is expected only when the side chain of N* contains the $>CH-CH_2CH_2-$ group. Thus total structure of N* can be deduced as 3-(3-amino-3-carboxypropyl)uridine (4abu^{3}U) (I).

Comparison of N with Authentic 4abu^{3}U.* Confirmation of the structural assignment of N* can be obtained by direct comparison of N* with synthetic 4abu^{3}U. For this purpose, 4abu^{3}U was chemically synthesized as described in the Materials and Results sections. Ultraviolet absorption spectrum of synthetic 4abu^{3}U was almost identical with that of N* as shown in Figure 2. Slight difference of ratio of A_{230} to A_{264} between N* and synthetic 4abu^{3}U may be due to the presence of contaminant in N* preparation, since it was eluted from the paper of the chromatogram. The mass spectrum of the trimethylsilyl derivative of synthetic 4abu^{3}U is identical with that of natural N*, showing in addition the presence of the higher derivative (pentasilyl) of molecular weight 705.

TABLE I: Relative Electrophoretic Mobilities of N*p and Acetic Anhydride Treated N*p.

	R_{Up} value	
	pH 2.0 (7% formic acid)	pH 7.5 (0.05 M triethylammonium bicarbonate)
N*p	0.55	0.88
Acetic anhydride treated N*p		1.03
Up	1.00	1.00
Cp	0.52	
Gp	0.60	0.88
G>p		0.56

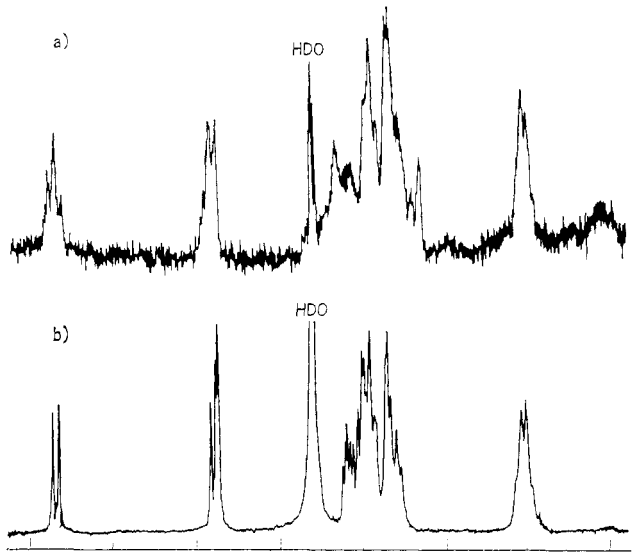


FIGURE 4: Nmr spectra of (a) N*p and (b) authentic 4abu^{3}U in D_2O. Scale represents 1 ppm.

This clearly indicates that N* is in fact 4abu^{3}U. Further proof that N* is identical with 4abu^{3}U was obtained from comparison of thin-layer chromatographic mobilities of N* with that of synthetic 4abu^{3}U. As shown in Table II, 4abu^{3}U behaved identically with N* in all solvent systems. As shown in Figure 4, the nmr spectrum of synthetic 4abu^{3}U was almost identical with that of natural N*p except for signals corresponding to protons at 2′ and 3′ positions of ribose.

Discussion

The modified nucleoside N*, located in the extra-region of *E. coli* tRNA^{Phe}, was thoroughly characterized as 3-(3-amino-3-carboxypropyl)uridine (4abu^{3}U). 4abu^{3}U can be also defined as an α-amino acid. The existence of a 3-amino-3-carboxypropyl group in a nucleoside is thus far unique, as no nucleoside with this side group has previously been found in material from nucleic acids. We have no data yet to determine directly the configuration of C-3 of the side chain. However, since authentic 4abu^{3}U which bears the L form in the above position behaved identically with N*, it is likely that natural N* is also in the L form. This assumption was also supported from *in vitro* synthesis of N* in tRNA using *S*-adenosyl-L-methionine as donor molecule for 3-amino-3-carboxyl group, as discussed later. It should be mentioned that several uracil derivatives having 2-amino-2-carboxyethyl group at N-1, C-5, or N-3 positions have been previously isolated from plants (Gmelin, 1954; Brown and Silver, 1966; Lambein and Van Parijs, 1968).

An unidentified modified nucleoside, designated as X, was also located in the same position in extra-region of *E. coli* tRNA^{Arg} (Murao *et al.*, 1971), tRNA^{Ile} (Yarus and Barrell, 1971), tRNA^{Met} (Cory and Marcker, 1970), and tRNA$_2^{Val}$ (Yaniv and Barrell, 1971). The 3′-phosphate of the modified nucleoside isolated from tRNA^{Arg} and tRNA^{Ile} had the same ultraviolet absorption spectrum as that of 4abu^{3}U, and behaved identically with it in two-dimensional thin-layer chromatography (Murao *et al.*, 1971; F. Harada and S. Nishimura, unpublished results). Therefore it is reasonable to conclude that the modified nucleoside found in these tRNAs is in fact 4abu^{3}U. On the other hand, an unknown modified nucleoside isolated from *E. coli* tRNA^{Met} seems to be different from 4abu^{3}U, since it behaved differently from it in two-dimensional chromatography. *E. coli* tRNA^{Phe} also contained a

TABLE II: Comparison of Thin-Layer Chromatographic Mobilities of N* with Authentic 4abu^{3}U.[a]

	R_F in Solvent System				
	1	2	3	4	5
N*	0.54	0.41	0.44	0.44	0.00
4abu^{3}U	0.54	0.41	0.44	0.44	0.00
U	0.55	0.67	0.56	0.52	0.25
C	0.68	0.46	0.47, 0.56	0.52	0.07
G	0.55	0.31	0.49	0.36	0.06
Up	0.43	0.80	0.43	0.11	

[a] The solvent systems used were described in the text.

derivative of 4abu^{3}U (N**) in relatively small amount as compared with 4abu^{3}U, as described in Materials and Methods. Its exact structure is not presently known, except that its ultraviolet absorption spectra are the same as 4abu^{3}U, and its molecular weight as shown by mass spectrometry is 44 mass units higher than 4abu^{3}U (Z. Ohashi and S. Nishimura, unpublished results). N** may be a derivative of 4abu^{3}U having a $C_2H_3(CHOHCH_3)CH(NH_2)COOH$ group instead of the 3-amino-3-carboxypropyl moiety. Exact nature of N** remains to be elucidated.

Characterization of the structure of the unique nucleoside, 4abu^{3}U, raises several interesting future problems, including its chemical modification and biosynthesis. 4abu^{3}U should be a very useful target for selective chemical modification of tRNA containing 4abu^{3}U, since several chemical reagents used for modification of amino acids can be used. In fact, Friedman (1973) recently reported that several uncharged *E. coli* tRNAs were eluted in the alcohol region of benzoylated DEAE-cellulose column chromatography if *E. coli* tRNA is treated with *N*-hydroxysuccinimide ester of phenoxyacetic acid, suggesting that N* present in these tRNAs is phenoxyacetylated. It is possible to specifically modify 4abu^{3}U by either a spin-labeling reagent or fluorescent substance to investigate the conformation of tRNA. The extra-region of tRNA containing both 7-methylguanosine and 4abu^{3}U must be very polar. The question of whether or not such a localized charged region plays a role in stabilizing a three-dimensional structure of tRNA is also of interest.

We were recently able to synthesize 4abu^{3}U in *E. coli* cell-free system using methyl-deficient tRNA^{Phe} as substrate and *S*-adenosyl-L-methionine as the donor molecule (S. Nishimura, Y. Taya, and Y. Kuchino, unpublished results). The transfer of the 3-amino-3-carboxylpropyl group from *S*-adenosyl-L-methionine without decarboxylation is a new type of enzymatic reaction. It has been also found that the enzyme which synthesizes 4abu^{3}U in tRNA is present in rat liver cell-free extracts (N. Okada, Y. Kuchino, and S. Nishimura, unpublished results). It is very likely that this enzyme is widely distributed in variety of organisms. In this respect, a search to detect 4abu^{3}U in tRNA from sources other than *E. coli* would be of interest. It was shown that mammalian tRNA contains 4abu^{3}U in large quantity (Randerath *et al.*, 1974). Friedman (1972) showed that certain species of rat liver tRNA reacted with *N*-hydroxysuccinimide ester of phenoxyacetic acid. This also suggests the presence of 4abu^{3}U in rat liver tRNA.

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