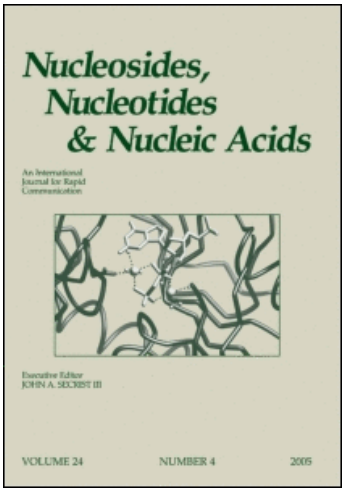

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ISOLATION AND CHARACTERIZATION OF A NOVEL NUCLEOSIDE FROM HUMAN CANCER URINE

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Abstract: A novel modified nucleoside, 5-carboxymethyluridine was isolated from 24 hour collection of urine from a lung carcinoma patient. The structure was assigned on the basis of UV, NMR and mass spectrometry, and confirmed by comparison of the spectral data and HPLC and paper chromatographic mobilities with those of the authentic sample. Possible origin and significance of this nucleoside is discussed.

Introduction

More than 15 modified nucleosides, derived primarily from tRNA[a] metabolism have been isolated from normal and pathological urines[1-3]. Included among these are the anticodon adjacent modified nucleosides N-[N-9-β-D-ribofuranosyl(purin-6-ylcarbamoyl)-L-threonine (t^6A)[4] and 1-methylinosine[3]. In addition to these nucleosides, a number of other modified nucleosides such as 2'-O-methyluridine[5] and N^2-dimethylguanosine[3] are also excreted routinely in human urine. Because there are no specific kinases to phosphorylate the modified nucleosides and also because there are no polymerases for incorporating their triphos-

[a]Abbreviations used are: tRNA - transfer ribonucleic acid, PFK - perfluorokerosine, TMS - trimethylsilyl, RP-HPLC - reversed phase high performance liquid chromatography.

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phatases into nucleic acids, these unusual nucleosides are either excreted intact in urine or metabolized to other reusable or excreted forms. This investigation reports the isolation and characterization of a novel nucleoside 5-carboxymethyluridine (I, Figure 1) from the urine of a lung carcinoma patient.

To our knowledge, this constitutes the first report of the presence of 5-carboxymethyluridine in urine. Even though the occurrence of this nucleoside has not been investigated in human tRNA hydrolysates, our findings suggest that 5-carboxymethyluridine or its methyl ester or amide is present in human tRNAs.

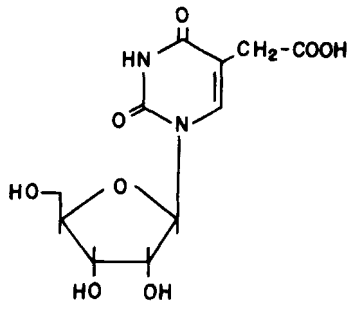
Materials and Methods

Neutral charcoal (Norit) was purchased from Fisher Scientific Co. Celite 545 was obtained from Johns-Mansville Co. and was washed with 6 N HCl, water, ethanol, and then dried before use. DEAE cellulose (DE-23) and AG-1X8 formate (200-400 mesh) anion exchange resin were obtained from Whatman and Bio-Rad Labs., respectively. Deuterium oxide (99.8 atom % D) was purchased from BioRad and HPLC grade methanol was obtained from Fisher Scientific. Deionized distilled water for use in HPLC was prepared in our laboratory. Acid washed papers (grade No. 589) were obtained from Schleicher and Schuell.

An authentic sample of 5-carboxymethyluridine was obtained from Dr. John D. Fissekis, Sloan Kettering Institute, New York, NY.

Ultraviolet spectrophotometry. Ultraviolet spectra were recorded on a Cary 219 spectrophotometer which was zeroed with water using the autobaseline feature. Spectra were determined with a scan rate of 0.2 nm/sec, absorbance range of 0.2-1.0 as full scale, and period of 1.0 sec.

NMR spectrometry. NMR spectra were determined on a Bruker WP-200 (200 MHz) spectrometer by utilizing the Fourier-transform/quadrature phase detection mode. Sample temperatures were maintained at 30° with a BVT-2000 temperature controller of the WP-200. Unless stated otherwise, the chemical shifts reported here are given in (δ) ppm, and are measured from internal TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-d4). The urinary unknown and the authentic sample were first lyophilized three times and then dissolved in 99.8% D_2O prior to NMR analysis.



(I)

Fig. 1. 5-Carboxymethyluridine.

Mass spectrometry. Initial mass spectra were acquired on a Finnigan 4000 mass spectrometer. Both the low and high resolution mass spectral data presented here were obtained using a Varian MAT 731 instrument with an ionizing energy of 70eV and a source temperature of 250°C. Sample introduction was via the direct probe inlet with removal of the solvent and reagents in the vacuum lock prior to insertion. The exact mass of unknown I was determined by peak matching (R=10,000) using PFK as the reference standard.

Chemical derivatization. Trimethylsilylation of the urinary unknown I and the synthetic standard was carried out by heating approximately 5ug of vacuum dried material with N,O-bis(trimethylsilyl)trifluoroacetamide, trimethylchlorosilane and pyridine (Supelco Inc., Bellefont, PA; (100:1:50)) in a sealed 3mm i.d. glass tube for 1 hour at 125°C.

Chromatography.

HPLC. High performance liquid chromatography (HPLC) was carried out on Altex Model 332 gradient liquid chromatograph, equipped with an Altex model 420 system controller programmer and an Altex model 153 analytical U.V. detector with a 8 µl flow cell set at wavelength 254 nm, and with the C-RIA Altex integrator. An analytical ODS-C18 column (5 µm Ultrasphere 0.46 x 25 cm) fitted with a 250 µl loop injector was used for coinjection studies. The HPLC solvent systems are described under Table 1. A semi-preparative Ultrasphere ODS C18 column (1.0 x 25 cm) 5 µm pore size fitted with a 2.0 ml loop injector and a preparative Zorbax

TABLE 1

Comparison of HPLC Retention Times (Minutes) of Unknown Urinary Nucleoside I and the Authentic 5-Carboxymethyluridine

Compound	Solvent Systems[a] I	II
Unknown Urinary Nucleoside I	8.23	24.44
5-Carboxymethyluridine	8.24	24.58
Urinary Nucleoside I + 5-Carboxymethyluridine	8.18	24.48

[a]Solvent Systems: (I) H_2O, 0.3 ml/min; (II) 0.1 M ammonium formate, pH 4.0, 0.7 ml/min.

column ODS C18 (2.12 x 25 cm) 8 μm pore size fitted with a 5.0 ml loop injector were also used.

Chromatography solvents for paper chromatography. Descending paper chromatography was performed in solvent saturated chambers using the following solvent systems v/v: (a) 2-propanol:water:conc. NH_4OH (7:2:1); (b) ethylacetate:2-ethoxyethanol:16% formic acid (4:1:2) (upper phase); (c) 1-butanol: glacial acetic acid:water (4:1:2); (d) 1-butanol: water:conc. NH_4OH (86:14: 5); (e) 1-propanol:water (60:40).

Isolation of the unknown urinary nucleoside I. The 24 hour pooled urine (1260 ml) from a lung carcinoma patient was passed through a charcoal-celite column (3.8 x 50 cm) (50 g each). The column was washed with water (2 liters) to remove salts and column bound material was eluted with 3 liters of 2 N NH_4OH in 50% aqueous ethanol. The eluate was evaporated to dryness (5.3 g), dissolved in 20 ml H_2O, and applied to a column (3.8 x 50 cm) of 200 g of AG-1X8 formate resin. The column was washed with 1.6 liters of H_2O and the wash was evaporated (2.8 g), dissolved in 10 ml H_2O, and then applied to a column (3.8 x 50 cm) of DEAE cellulose-23 (50 g) equilibrated with 0.14 M boric acid[6]. To separate the nucleoside fraction from other components, the column was first washed with 1.1 liters of 0.14 M boric acid and then eluted with 1.5 liters of 0.7 M boric acid. The 0.7 M boric acid fraction was

evaporated to dryness after repeated addition of methanol (4 x 100 ml) and lyophilized (127 mg). The lyophilate was dissolved in 3 ml H_2O, centrifuged, filtered, and passed through a Zorbax preparative reversed phase HPLC column using a 0 → 25% in 60 min methanol gradient in 0.1 M ammonium acetate buffer, pH 7. Of the 26 fractions separated on this column, fraction 10 (48 A_{260} units) was passed through an Ultrasphere semi-preparative reversed phase HPLC column (1.0 x 25 cm) and separated into 10 fractions using a 0 → 25% in 75 min methanol gradient in H_2O after an initial 30 min H_2O wash. Fraction 5 (9 A_{260} units) of these 10 fractions was purified to homogeneity by another passage through the semi-preparative reverse phase column using 0.1 M ammonium formate buffer, pH 4.0. The unknown urinary nucleoside (I) had UV spectra similar to other C5-substituted uridines with λ_{max} (nm) at 265 (pH 1.7 and 11.8) and 266 (pH 6.3) (Figure 2). This material was used for mass spectral and nmr analyses as well as for high-performance liquid chromatographic studies.

Results

As stated above the UV spectra of the urinary unknown nucleoside I demonstrated absorption maxima at 265 nm (pH 1.7 and 11.8) and 266 nm (pH 6.3) (Fig. 2a). These maxima are characteristic of several 5-alkyl substituted uridines; however, 5-alkoxyuridines such as 5-methoxyuridine absorb maximally at 275 nm in acidic, neutral and alkaline pH's. In NMR spectrum, the urinary compound exhibited a singlet at 7.73 ppm for the C_6 proton of uracil moiety while there was no signal for the C_5 proton (Table 2). There was a doublet centered at 5.97 ppm for the anomeric proton of the sugar carbon $C_{1'}$ which together with the signals observed from the other sugar carbon protons, indicates the presence of a ribose. A singlet is observed at 3.23 ppm with an intensity approximately twice that of the C_6 signal suggesting the presence of a CH_2 group. The chemical shift and the coupling constant (4.4 Hz) for the anomeric proton in nmr spectra suggested that the natural material was in β-configuration[7,8]. The other naturally occurring nucleosides, 5-methyluridine, 5-hydroxymethyl uridine and its deoxy isomer, and orotidine were readily eliminated on the basis of these nmr spectra. The uv (fig. 2b) and nmr spectra of the authentic 5-carboxymethyluridine[9] were similar to those of the naturally occurring material.

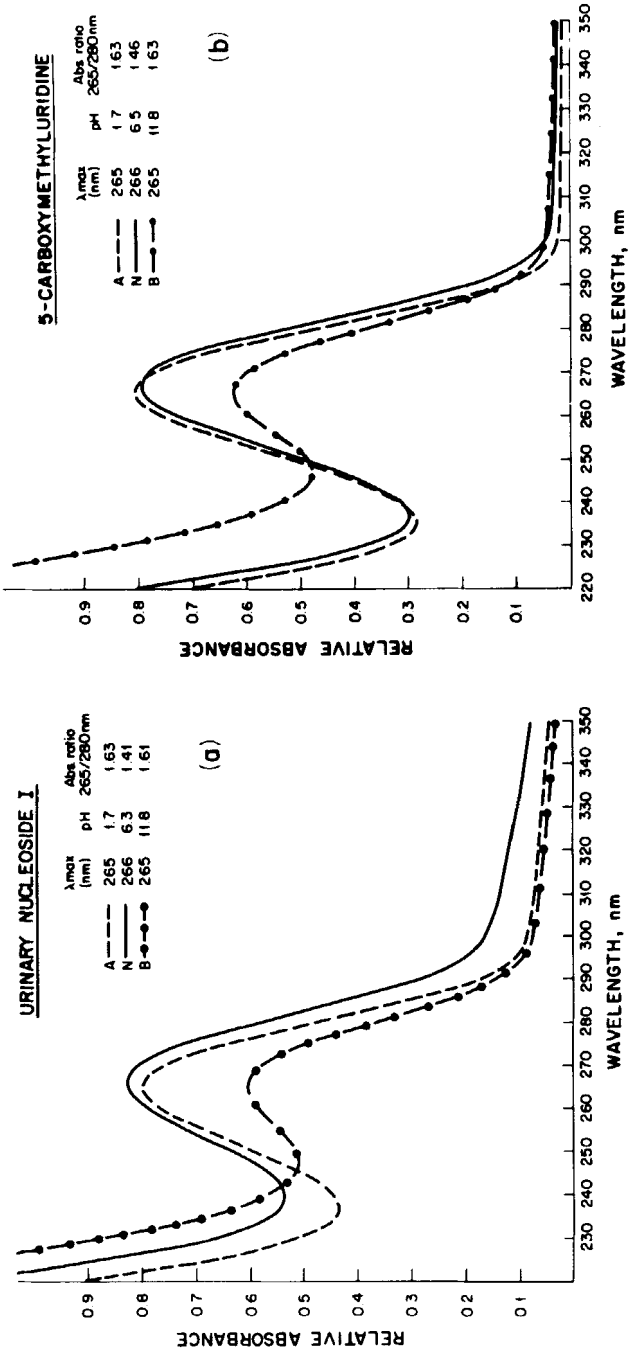


Fig. 2. Ultraviolet spectra of (a) urinary nucleoside, and (b) authentic 5-carboxymethyluridine.

Table 2
Chemical Shifts and J Values for Unknown Urinary Nucleoside and Related Compounds in D_2O

Compound	C_6-H	C_1'-H (J)	C_5-substituent
Urinary Nucleoside	7.73	5.98 (4.4)	3.22 (CH_2)
5-Carboxymethyluridine	7.73	5.97 (4.5)	3.22 (CH_2)
5-Methyluridine	7.71	5.95 (4.6)	1.92 (CH_3)
Orotidine	--	5.59 (3.4)	5.80 (H)

Urinary unknown I was converted to its TMS derivative for both low and high resolution mass spectrometric analysis. The assignments shown in figure 3a are based on the fragmentation processes of nucleosides as discussed by Pang et al.[10] Exact mass analysis was of the tetra-TMS derivative since that species was predominant. A penta-TMS derivative was also observed but not used since its abundance was too low to give a satisfactory result. The measured mass of 590.2314 corresponded to the molecular composition $C_{23}H_{46}O_8N_2Si_4$ (calc. mass 590.2331) suggesting the underivatized species to be 5-carboxymethyluridine ($C_{11}H_{14}O_8N_2$) or isomeric to it.

A molecular ion of m/z 662 was observed in the low resolution spectrum (fig. 3a) with a more intense M-CH_3 at m/z 647. This derivatization was independent of that used for the exact mass determination and showed the penta-TMS species almost exclusively. The sugar ion series (m/z 169, 217, 230, 243, 245, 258, 259, 348 $[S-H]^+$, 349)[10] corresponds to that of the pentose sugar accounting for three of the five TMS moieties. The remaining two are taken up by the 4-O of uracil and the free carboxyl group; the latter being supported by an intense ion at m/z 117 (CO_2TMS). The base series of ions (m/z 299, 313, 314, 315 $[BH_2^+]$, 326, 343, 354, 371, 387, 415, 429, 445)[10] is observed as well.

The low resolution mass spectrum of unknown I was compared with that of the synthetic standard (fig. 3b). The spectrum of the isolated material exhibits ions from low molecular weight impurities, but with respect to the structurally diagnostic ions the spectra are experiment-

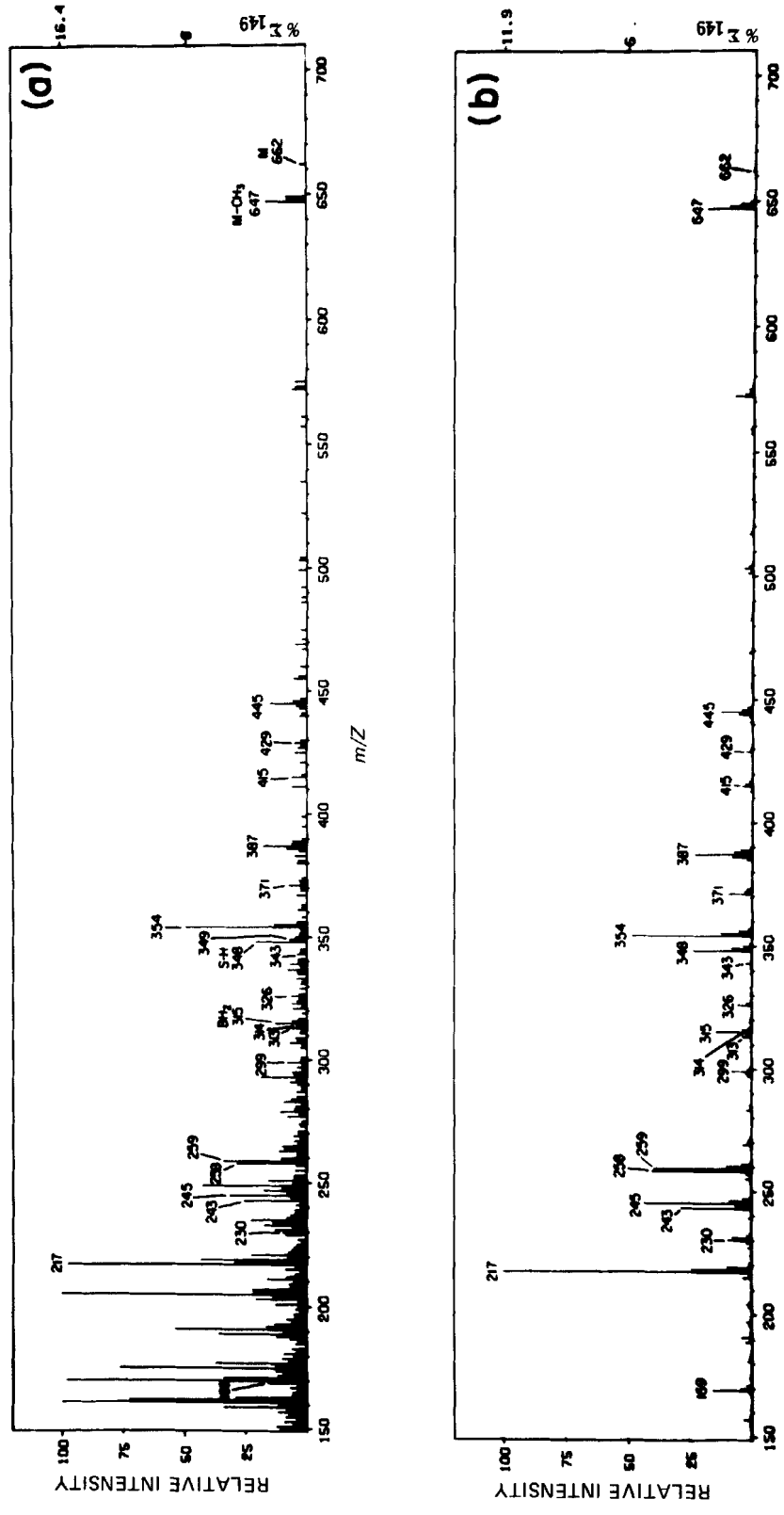


Fig. 3. (a) Mass spectrum of trimethylsilyl derivative of urinary unknown I. (b) Mass spectrum of the trimethylsilyl derivative of authentic 5-carboxymethyluridine $(TMS)_5$.

TABLE 3

Paper Chromatography Comparison of the Unknown Urinary Nucleoside I with Authentic 5-Carboxymethyluridine and Uridine

	R_f X 100 Solvent Systems[a]				
Compound	a	b	c	d	e
Unknown Urinary Nucleoside	39	35	42	0.5	57
5-Carboxymethyluridine	39	35	42	0.5	57
Uridine	45	37	46	7	69

aSolvents are described in Materials and Methods section.

ally the same, indicating the identity of unknown I to be 5-carboxymethyluridine.

In order to confirm the identity further, the unknown urinary nucleoside (I) was coinjected with the authentic 5-carboxymethyluridine on RP-HPLC column and was eluted as a single peak in two solvent systems (Table 1). Paper chromatographic mobilities of the urinary unknown were also identical to those of the authentic sample in five solvents (Table 3).

Discussion

The new urinary nucleoside 5-carboxymethyluridine (I) was isolated in 0.34 mg from the 24 hour collection of urine from a lung carcinoma patient. The presence of this nucleoside in alkaline hydrolysates of yeast and wheat embryo tRNA has been reported[11,12]. Enzymatic hydrolysis of these tRNAs under neutral conditions revealed that methyl ester and amide derivatives of carboxymethyluridine are present rather than the free acid[13,14]. Sequence studies indicated that the methyl ester of carboxymethyluridine occurs in the wobble position of $tRNA_{Arg-3}$ from yeast[13]. In terms of the function of this nucleoside in tRNA, it is suggested that at a polynucleotide level the 5-substituent may have the modulating influence on the conformation and interaction of the anticodon loop[8]. The 5-methylcarboxymethyl group in

methyl ester of I may prevent its wobble with guanine. This nucleoside like a number of other urinary modified nucleosides might originate from the catabolism of tRNA. Because of the lack of appropriate kinase and polymerase it cannot be recycled into tRNA, and as a result it is excreted into urine.

Previously isolated, transfer RNA-derived urinary nucleosides such as 1-methylinosine, N^2-dimethylguanosine[3] and pseudouridine have served as indicators of tumor burden in cancer patients[15-18]. Five normal urines when investigated for modified nucleosides, did not show the presence of 5-carboxymethyluridine or its methyl ester. The presence of this compound in lung carcinoma patients and its significance as a potential tumor marker is being investigated.

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