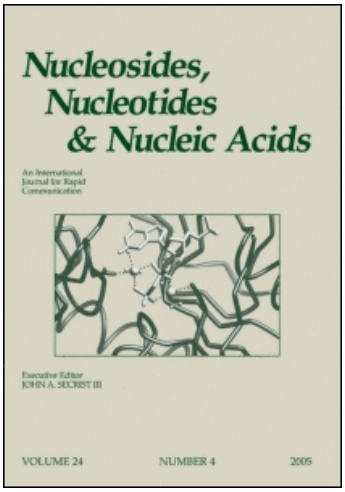

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IDENTIFICATION AND SYNTHESIS OF 5'-DEOXYXANTHOSINE, A NOVEL NUCLEOSIDE IN NORMAL HUMAN URINE1

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ABSTRACT

Methods used in the identification and synthesis of 5'-deoxyxanthosine (5'-dX), a novel nucleoside found in normal human urine, are described. The urine sample was separated into nucleoside components by HPLC. Low and high resolution GC/MS techniques were then used to assign a tentative structure of the new nucleoside as 5'-dX. Finally, after biochemical synthesis, a comparison of the reference material with the urinary sample using mass spectrometry confirmed the structure as 5'-dX.

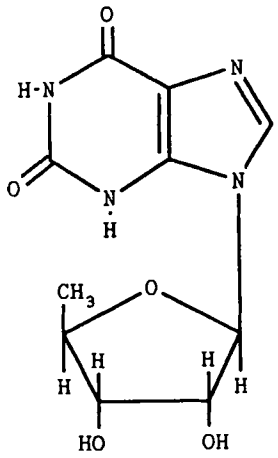
INTRODUCTION

Modified nucleosides occurring in human urine have been widely studied as possible "biomarkers" of cancer[2,3] and certain immunodeficiency diseases[4], including AIDS.[5,6] The basic rationale for such work is that rapid catabolism of t-RNA in tumor cells results in increased urinary excretion of modified nucleosides which are not amenable to reuse. For example, a correlation has been suggested between the presence of cancer and elevated levels of pseudouridine (ψ), 1-methyladenosine (m^1A), 1-methylinosine (m^1I), N^2-methylguanosine (m^2G) and N^2,N^2-dimethylguanosine (m_2^2G).[2,3,7-9] In addition, a connection between elevated levels of these nucleosides and the degree of tumor involvement or response to therapy

has been established.[7,8] In one study,[9] urinary levels of ψ, m^1I and m_2^2G decreased with a positive response to therapy and increased with treatment failure. Alternatively, another study[10] found no correlation between levels of ψ, m^1G, m^2G or m_2^2G in the urine of patients with gastro-intestinal cancer when the levels of these compounds were determined in pre- and post-operative samples.

A second aspect in studying urinary nucleosides is the identification and quantitation of nucleosides not related to t-RNA, but which derive from other biochemical processes that may or may not be related to cancer or other disease states. In this category is 7-β-D-ribofuranosyl-hypoxanthine, the first N7-glycosidically - linked nucleoside to be found in human urine.[11] This novel nucleoside was identified in the urine of a patient with chronic myelogenous leukemia (CML) and is thought to originate from the abnormally high levels of vitamin B_{12} characteristic of this disease - a logical view-point since this compound is not found in normal or non-CML cancer patient urine. Other examples of human urinary nucleosides not originating from t-RNA include N^6-succinyladenosine, excreted at elevated levels in the urine of patients with metastatic disease,[12,13] and 1-[β-D-ribofuranosyl]-pyridin-4-one-3-carboxamide, a compound found in the urine of CML patients at lower than normal levels.[14]

As part of a program to identify and quantitate modified nucleosides in the urine of cancer patients and to compare the excretion levels with normal values, we report the identification of 5'-deoxyxanthosine (5'-dX, **1**) in normal human urine. Nucleoside **1** was partially purified using boronate gel affinity chromatography and reversed phase HPLC and characterized by GC/MS techniques. Structure proof is based on a comparison of the urinary nucleoside with a reference sample prepared using biochemical synthetic procedures.



1,5'-Deoxyxanthosine

MATERIALS AND METHODS

Chemicals and Chromatographic Standards

Methanol (HPLC grade), ammonium acetate buffer and formic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 2'-Deoxyguanosine (dG), 2'-deoxyadenosine (dA), 3'-deoxyadenosine (3'-dA), 5'-deoxyadenosine (5'-dA), purine nucleoside phosphorylase (PNP, N-3003, from bovine spleen. and xanthine oxidase (XO; X1875) were purchased from Sigma Chemical Co. (St. Louis, MO). 3'-Deoxyguanosine (3'-dG) was obtained from Yamasa Shouyu Co. Ltd. (Tokyo, Japan). Water was purified with a Milli-Q Reagent water system (Millipore, Bedford, MA).

Urine Collection and Chromatographic Isolation

An 80 mL sample of urine from a normal subject (KN) was collected following a 12-hour fasting period. The urine sample was centrifuged to remove particulate matter and stored at -20° until needed.

Boronate gel affinity chromatography used to isolate the urinary nucleosides was a modification of a published procedure.[15] Affi-gel 601 (Bio-Rad Labs., Richmond, CA) with a specific affinity for cis-hydroxyl groups was packed in a column (60 x 9 mm I.D.; bed volume 0.83 mL) equilibrated with 20 mL of 0.25 M ammonium

acetate (pH 8.8) and washed with 20 mL of 0.1 M formic acid. A 10 mL aliquot of urine was adjusted to pH 8.8 with 2.5 M ammonium acetate (pH 9.5) and loaded onto the column (previously equilibrated with 0.25 M ammonium acetate). The column was then washed with 8 mL of 0.25 M ammonium acetate (pH 8.8) and the nucleosides eluted with 4 mL of 0.2 M formic acid. The eluate was evaporated to dryness under reduced pressure and redissolved in 1 mL of water for HPLC purification.

A Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisting of an LC-6A system controller and dual pumps, a SPD-6AV UV-Vis detector and a C-R3A Chromatopac integrator with recorder was used in the isolation of the individual nucleoside fractions. Analytical and preparatory HPLC procedures used 5 μm Develosil ODS-5 reversed phase column (250 x 4.6 mm ID) (Nomura Chemicals, Nagoya, Japan) and a 15-30 μm Develosil ODS-5 pre-column. A filter (1 μm mesh size; Nomura Chemicals) was installed between the injector and pre-column.

Separation of the urinary nucleoside components was performed starting with ultra-pure water (pH 4.2, adjusted with 0.2 M formic acid) for 25 minutes at which time the mobile phase was changed to 13:87 methanol-water (v/v). At 35 minutes the mobile phase consisted of 45:55 methanol-water (v/v) and, finally, at 40 minutes, the column was washed with water (pH 4.2). The flow rate was 1.1 mL/min with an injection volume of 300 or 350 μL. Using UV detection (260 nm), a total of 22 peaks were fractionated as possible urinary nucleosides. By comparing retention times and UV absorption spectra of the urinary components to standard samples, tentative identification of the major component(s) of each fraction was made. The material from each of the 22 components was collected and lyophilized for GC/MS analysis.

Gas Chromatography and Mass Spectrometry Conditions

Each lyophilized HPLC fraction was reconstituted in water to a concentration of 1 μg/μL and a 10 μL aliquot

taken to dryness under a stream of nitrogen. The trimethylsilyl (TMS) derivative of the nucleoside fraction was then prepared using standard methods.[16] A 1 μL aliquot of the derivatization reaction mixture was injected onto the GC column for analysis.

GC/MS analyses were performed using a Varian 3400 gas chromatograph fitted with a DB-5 capillary column (30 m x 0.25 mm, 0.25 μm film thickness) directly coupled to a Finnigan MAT90 mass spectrometer (Finnigan Corp., San Jose, CA).

GC Conditions: initial temperature, 150°C; program rate, 6°C/min to 300°C with a final hold time of 5 min; carrier gas was He at a head pressure of 10 psig; injector was splitless at 300°C; and the interface temperature was 300°C.

Mass Spectrometer Conditions: conditions for low resolution electron impact (EI) analysis were as follows: source temperature, 250°C; mass range scanned from m/z 70 to 1000 at a scan rate of 0.4 seconds/decade and resolution of 1000 (10% valley). Mass spectrometer control, data acquisition and data storage used a Micro VIP 11/73 computer (US Design, Palo Alto, CA).

High resolution measurements on the reference sample were performed using a scan rate of 10 seconds/decade at a resolution of 7500 (10% valley definition) and sample introduction by direct insertion probe.

Preparation of Reference Samples

A reference sample of 5'-deoxyinosine (5'-dI) was prepared by deamination of 5'-dA with sodium nitrite.[17] The product was purified and isolated using HPLC as described above and the structure of the sample confirmed by comparison of the mass spectrum of the TMS derivative with data from the literature.[18]

5'-dI was enzymatically cleaved to provide 5-deoxyribose-1-phosphate (5-dR-1-P) using a modification of a literature method.[19] The reaction mixture for the preparation of 5-dR-1-P contained 0.6 mmole 5'-dI, 2.5 units of

PNP, 0.125 units of XO and 50 mM potassium phosphate buffer (pH 7.5 adjusted using 5N sodium hydroxide), in a total volume of 5 mL. Prior to the reaction, the two enzymes had been dialyzed against 50 mM phosphate buffer (pH 7.5) to remove ammonium sulfate. In order to minimize loss of activity, the enzymes were not immobilized as originally reported.[19] The reaction was allowed to proceed for 12 hours at 23°C on a rotary shaker. Following reaction the enzymes and inorganic phosphate were removed by membrane filtration and precipitation using barium hydroxide and barium acetate, respectively. The mixture containing 5-dR-1-P was used without further purification in the synthesis of 5'-deoxyguanosine (5'-dG).

Preparation of 5'-deoxyguanosine (5'-dG) was performed[19] by reaction of 140 μmol of guanine with 80 μmol of 5-dR-1-P in the presence of 4 units of PNP in 10 mM Tris-HCl (pH 7.5) in 20 mL total volume. The reaction was carried out at 23°C for 5 hours on a rotary shaker. 5'-dG was isolated using reverse phase HPLC and the structure of the product confirmed by GC/MS analysis. 5'-dG was then deaminated using sodium nitrite as described earlier.[17] Attempts to directly couple xanthine to 5-dR-1-P using PNP were unsuccessful.

Reference samples of 2'-deoxyxanthosine (2'-dX) and 3'-deoxyxanthosine (3'-dX) were prepared by deamination of the respective deoxyguanosines using sodium nitrite.[17] Products were isolated and purified using HPLC and structures confirmed by GC/MS.

RESULTS AND DISCUSSION

Low Resolution Mass Spectrometry

The TMS derivatives of the 22 HPLC purified urinary nucleoside fractions were screened, using GC/MS, to verify the presence of components suspected as being present based on HPLC retention times and UV absorption spectra. Most of the fractions contained, in addition to

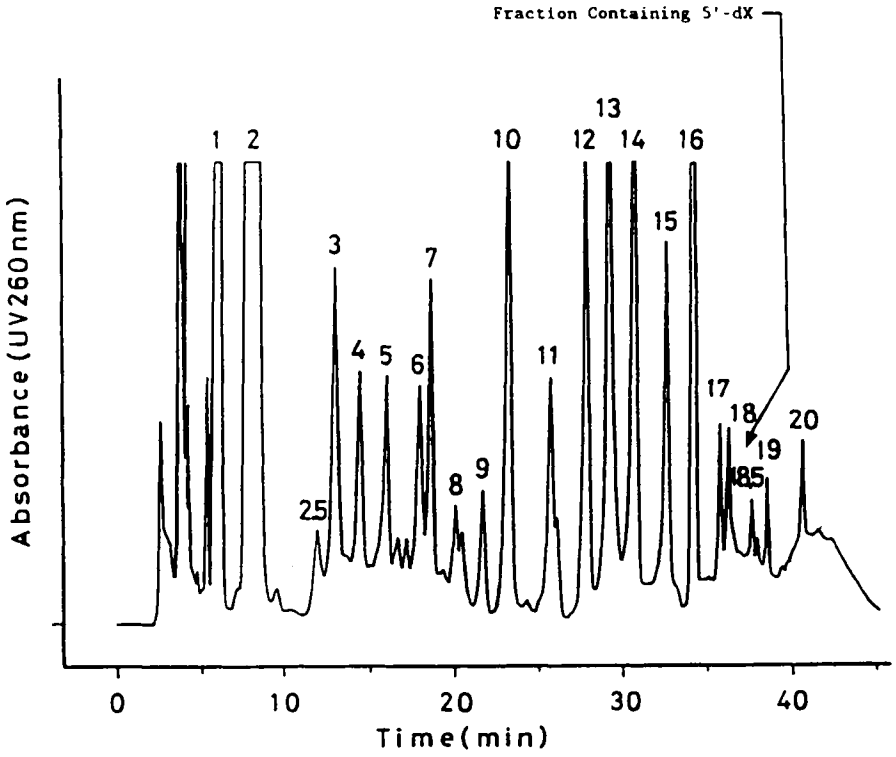


Figure 1. HPLC elution profile of normal human urine. UV detection at 260 nm.

known components, nucleosides of unknown structure. A close inspection of the reconstructed ion chromatogram and mass spectra from fraction 18.5, i.e., the HPLC fraction having a retention time of 37.60 minutes (see Figure 1 for HPLC elution profile), revealed what appeared to be a nucleoside of unknown structure, designated UNK 18.5-1, in low concentration with a GC retention time of 22.73 minutes. [From the HPLC retention time and UV absorption spectra, fraction 18.5 was thought to contain only 2-methylthio- N^6-isopentenyladenosine (ms^2i^6A).] UNK 18.5-1 appeared as one of five unidentified nucleosides[20] in this fraction.

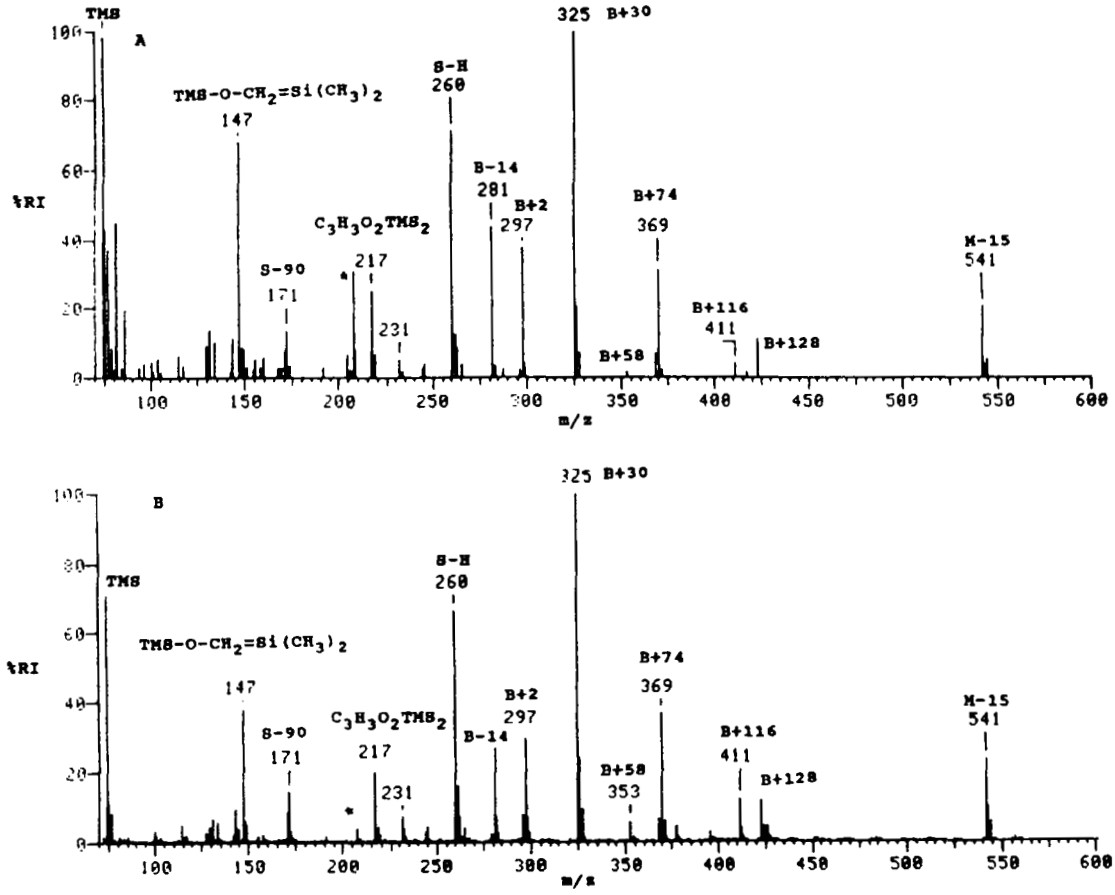


Figure 2. Low Resolution EI (70 eV) spectra of the TMS derivatives of a) urinary component and b) reference sample of 5'-dX (*indicates the presence of background ions).

The EI spectrum of UNK 18.5-1, shown in Figure 2, contained the following important features:[21-23]

a. Molecular weight related ions. The molecular weight of the sample was tentatively assigned as 556 daltons(d), even though a molecular ion was not observed, on the basis of an M-15 ion (m/z 541, 21%).

b. Ions related to the sugar. The presence of a modified sugar was suggested by the absence of ions normally

observed in the spectrum of ribonucleosides. Instead, ions associated with a deoxypentose functionality were observed and were assigned as follows: S-H (m/z 260, 71%), $C_3H_3O_2$TMS (m/z 217, 26%), S-TMSOH (m/z 171, 14%), S-H-TMSOH (m/z 170, 12%), C_3H_4OTMS (m/z 129, 6%) and CH_2OTMS (m/z 103,3%). The structure of UNK 18.5-1, therefore, appeared to contain a deoxypentose sugar, with the site of dehydroxylation being at the 2'-, 3'- or 5'-positions. The reduced intensity of the m/z 103 ion, relative to the intensity of this ion in the spectra of ribosides and 2'- and 3'-deoxynucleosides,[21-23] suggested the presence of a 5'- deoxyribose, but this data alone was not conclusive.

c. Base ion series. Subtraction of the mass of a deoxypentose residue from the molecular ion gave a difference of 295 d, which was the mass assigned to the aglycone and confirmed by the presence of an ion at m/z 297 (12% RI) for the B+2H ion of UNK 18.5-1. The presence of two TMS groups in the sugar and two in the base residue was established by preparation of the TMS-$^2H_9$ derivative.[24] The mass spectrum of the TMS-$^2H_9$ derivative showed an appropriate shift in diagnostic ions for this distribution of blocking groups based on the tentatively assigned structure (Table 1). Additional peaks which strengthened the assignment of a weight of 295 d to the aglycone included the following ions: B+128 (m/z 423, 8%), B+116 (m/z 411, 5%), B+100 (m/z 395, 3%), B+74 (m/z 369, 37%), B+58 (m/z 353, 2%), B+30 (m/z 325, 100%) and B-14 (m/z 281, 44%). The structures and significance of these ions have been previously described.[21,22]

Subtraction of 144 d, two TMS groups, from 296 d, the weight of the aglycone plus a hydrogen, gave 152 mass units for the molecular weight of the free base which suggested the presence of xanthine. A comparison of the mass spectrum of UNK 18.5-1 with a library spectrum of xanthosine(TMS)$_5$[25] showed the presence of a number ions common to both spectra including the m/z 411, 369, 353, 325, 297, 281 and 217 peaks, with the m/z 325 ion being the

TABLE 1, Comparison of the Major Peaks in the Mass Spectra of the TMS and TMS-$^2H_9$ Derivatives of Urinary 5'-Deoxyxanthosine:

m/z (TMS)	m/z (TMS-$^2H_9$)	Shift In AMU	No. of TMS Groups Indicated	Structural Assignment (ref. 21,22)
541	547	33	3+$(CH_3)_2$	M-15
423	450	27	3	B+128
369	369	27	3	B+74
325	343	18	2	B+30
297	315	18	2	B+2H
281	296	15	1+$(CH_3)_2$	B-14
260	278	18	2	S-H
217	235	18	2	$C_3H_3O_2TMS_2$
171	179	8	1-H	S-TMSOH

base peak in both spectra above m/z 100. The spectra of the two compounds did, however, differ in the presence of typical riboside peaks in the spectrum of xanthosine(TMS)$_5$. Thus, UNK 18.5-1 was tentatively assigned a structure containing xanthine as the aglycone which was coupled to a deoxypentose.

Additional information as to the site of dehydroxylation in the sugar was available from other ions in the low resolution mass spectrum. For example, the presence of a hydroxyl group at the 2'-position was strongly indicated by the presence of the B+116 ion, known from earlier studies[21-23] to contain the C-1', C-2' and O-2' portions of the sugar ring. Likewise, a hydroxyl in the 3'-position was suggested by the B+128 peak.[23] Studies of purine and pyrimidine nucleoside TMS derivatives labeled in specific positions with ^{18}O indicate[21] the OTMS portion of the B+128 ion contains the 3'-hydroxyl group. Thus, the only position remaining for removal of a hydroxyl group is the 5'-position and a tentative

TABLE 2, Elemental Composition and Structural Assignments for Significant Ions in the Mass Spectrum of 5'-dX:

m/z (Observed)	Elemental Composition (Error in millimass units, mmu)		Structural Assignment (ref.[21,22])
541.2128	$C_{21}H_{41}N_4O_5Si_4$	(-2.6)	M-15
369.1562	$C_{14}H_{29}N_4O_2Si_3$	(-3.6)	B+74
353.1253	$C_{13}H_{25}N_4O_2Si_3$	(-3.2)	B+58
325.1147	$C_{12}H_{21}N_4O_2Si_2$	(-0.5)	B+30
297.1203	$C_{11}H_{21}N_4O_2Si_2$	(0.0)	B+2H
281.0891	$C_{10}H_{17}N_4O_2Si_2$	(0.1)	B-14
260.1250	$C_{11}H_{24}O_3Si_2$	(-1.4)	S-H
217.1076	$C_9H_{21}O_2Si_2$	(-0.4)	$C_3H_3O_2TMS$
171.0834	$C_8H_{15}O_2Si$	(-0.7)	S-90

structure for UNK 18.5-1 was assigned as 5'-deoxyxanthosine.

High resolution mass measurements.

Table 2 contains the results of high resolution mass measurements which established the elemental composition of the major ions in the mass spectrum of biosynthetically prepared 5'- dX. This data confirmed the tentative assignments made based on the low resolution mass spectral data.

Comparison Studies of 2'-, 3'- and 5'-Deoxyxanthosine

Initially, difficulty was anticipated in the chemical synthesis of a reference sample of 5'-dX because the required precursor, 5'-deoxyguanosine (5'-dG), is not commercially available. Although numerous methods for the chemical preparation of 5'-deoxynucleosides are available,[26,27] including 5'-dG,[28] the procedures are multistep reactions requiring an advanced level of synthetic expertise in nucleoside chemistry. An alternative route was investigated which involved a more straight forward preparation of reference samples of 2'-dX and 3'-dX by a

one-step deamination reaction[17] of commercially available dG and 3'-deoxyguanosine(3'-dG). These two reference materials were prepared in the hope that a comparison of the chromatographic and mass spectral characteristics of 2'- and 3'-dX[29] with those of UNK 18.5-1 would establish the identity of the unknown as one of these two samples, or eliminate either of these compounds as being identical with the unknown.

HPLC retention times for 2'-dX, 3'-dX and 5'-dX are shown in Table 3. The HPLC peak for the biochemically prepared 5'-dX was very broad with a retention time of 36.21 min. The broad peak may be a consequence in 5'-dX and ODS interactions. The UV absorption data of 2'-dX, 3'-dX and 5'-dX in water (pH7) (Table 3) show the double maximum similar to xanthosine.

The GC/MS retention times for 2'-dX, 3'-dX, 5'-dX and UNK 18.5-1 are also included in Table 3.

The comparison of the mass spectra and retention times of the reference materials of the TMS derivatives of X, 2'- and 3'-dX with those of UNK 18.5-1 showed significant differences in both the mass spectra and retention times which strengthened the assignment of UNK 18.5-1 as being 5'-dX. A subsequent study of differences in the mass spectra of a series of 2'-, 3'- and 5'-deoxynucleoside TMS derivatives[29] revealed that the structure of these isomeric compounds can be readily assigned based on mass spectral differences, even if only one compound is available for analysis.[30,31] However, ultimate proof of structure required preparation of a reference sample of 5'-dX for a direct comparison of the GC retention time and mass spectral fragmentation pattern with that of the unknown.

Comparison of the Mass Spectra of Reference 5'-dX(TMS)$_4$ With the Urinary Component

A sample of the reference 5'-dX was prepared and the GC and mass spectral behavior of this sample compared with UNK 18.5-1. The GC retention time for the urinary component was essentially identical with the retention time of

TABLE 3, UV Absorption Spectra and Chromatographic Retention Time Data for Reference Samples of X, 2'-, 3'-, and 5'-dX and the Urinary Component UNK 18.5-1.

Compound	UV Spectrum (H_2O, pH 7) λ max	(Abs)	HPLC Retention Time (minutes)	GC Retention Time (min) TMS Deriv.
X	248nm	(10.2)[a]	N/A	25.12 (TMS_5)
	278nm	(8.9)[a]		
2'-dX	246nm	(0.54)	36.94	25.13 (TMS_4)
	284nm	(0.35)		
3'-dX	238nm	(1.84)	35.18	24.04 (TMS_4)
	262nm	(1.90)		
5'-dX	242nm	(1.69)	36.21	22.72 (TMS_4)
	257nm	(1.77)		
UNK18.5-1			37.60[b]	22.73 (TMS_4)

[a] These values are extinction coefficients (X 10^{-3}).

[b] The retention time of the urinary component is that of the major nucleoside in this fraction, i.e. m^6A. The minor component identified as 5'-dX has a retention time somewhere between fractions 18 and 18.5, but a more precise determination of the HPLC retention time of this urinary component is not possible at the present time.

the reference 5'-dX, as shown in Table 3. Figure 2 shows the comparison of the mass spectra of both of the samples; the spectra are seen to be essentially identical in both m/z values and relative abundance of the structurally significant ions.

The retention time data is important in eliminating the possibility of epimeric hydroxyl groups or the presence of anomers, since some difference in retention time would be expected with these structural variations.[16,32] In addition, of the cis-diol specific boronate column used in isolation of the urinary nucleosides decreases the possibility of sugars with an arabino or xylo

configuration being present in the riboside containing fraction, although the lyxo isomer is not excluded by this reasoning. (In fact, isomer, epimer or anomer problems are eliminated on the basis of the biosynthetic scheme used to prepare the 5'-dX reference sample.)

Likewise, ion abundance values would be expected to show noticeable differences if epimers or anomers were present.[21] Thus, on the basis of the biochemical synthetic scheme, identical GC retention times and the almost superimposable mass spectra of the reference material and urinary component, the structure of UNK 18.5-1 was established as 5'-deoxyxanthosine.

Finally, 5'-dX has been recently identified in a sample of pooled normal human urine. The pool consisted of 300 male and female subjects from the PL Osaka Health Control Center. The average level of urinary 5'-dX was estimated from spectral data at less than 0.40 μg/mL for the pool of normal urine sample. The total UV absorbance for the total HPLC fraction which included 5'-dX was 2.8 A_{257}. A more accurate determination of the 5'-dX was not possible since GC/MS analysis of the fraction revealed numerous components.

Possible Biochemical Origins of 5'-dX

5'-Deoxyxanthosine is only the second nucleoside of this novel class to be identified in human body fluids. The first compound of this class, 5'-deoxyinosine (5'-dI), was identified very recently in the urine of chronic myelogenous leukemia (CML) patients.[18] A possible source of 5'-dI in mammals involves 5'- deoxyadenosine (5'-dA), which has long been known to be present in vitamin B12[33], levels of which are abnormally high in CML. Cleavage of 5'-dA by 5'-methylthioadenosine phosphorylase produces adenine base and 5-dR-1-P.[34] The latter component, in the presence of hypoxanthine and PNP, may produce 5'-dI.[18] Using the same rationale, 5'-dX may be the product of PNP catalyzed condensation of 5-dR-1-P with xanthine. Xanthine is known to be excreted at a level of 70-90

μmol/day in human urine and to be present in serum in 1-20 μM concentration.[35] However, when the PNP catalyzed reaction of xanthine with 5-dR-1-P was performed during the course of attempts to prepare the reference material no 5'-dX was formed as determined by HPLC and GC/MS analysis of the reaction products. However, since the PNP used in this experiment was derived from bovine spleen and since the substrate specificity of the bovine PNP may differ from human PNP, no definite conclusions can be drawn concerning this potential biosynthetic route.

Another possible source of 5'-dX would be the oxidation of 5'-dI by xanthine oxidase (XO). Monitoring the reaction of 5'-dI with XO, however, eliminated this pathway as the source of 5'-dX since no 5'-dX was observed in the HPLC and GC/MS analysis of the reaction mixture. At the present time, the biochemical origin of 5'-dX remains unknown and work is in progress to determine the origin of this new and novel nucleoside isolated from normal human urine.

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