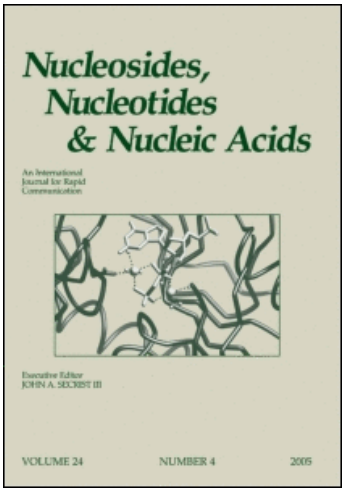

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Isolation and Characterization of an Unusual Nucleoside, 1-α-D-Ribofuranosyl-4-pyridone-3-carboxamide, from the Urines of Normal Human Individuals and Leukemic Patients

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ISOLATION AND CHARACTERIZATION OF AN UNUSUAL NUCLEOSIDE, 1-α-D-RIBOFURANOSYL-4-PYRIDONE-3-CARBOXAMIDE, FROM THE URINES OF NORMAL HUMAN INDIVIDUALS AND LEUKEMIC PATIENTS[a]

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Abstract: A novel modified nucleoside, 1-α-D-Ribofuranosyl-4-pyridone-3-carboxamide, has been isolated from the urines of one normal human individual and two CML patients. The structure was assigned on the basis of UV, NMR and mass spectral data and confirmed by comparison with an authentic sample synthesized in our laboratory. This constitutes the first example of the occurrence of a nucleoside with an α-riboside linkage in human urine.

Abbreviations used are: tRNA, transfer ribonucleic acid; β-4-PCNR, 1-β-D-ribofuranosyl-4-pyridone-3-carboxamide; NAD, nicotinamide adenine dinucleotide; α-4-PCNR, 1-α-D-ribofuranosyl-4-pyridone-3-carboxamide; CML, chronic myelogenous leukemia; TMS, trimethylsilyl; RP-HPLC, reversed phase high performance liquid chromatography; HMDS, Hexamethyl disilazane; TMCS, trimethyl chlorosilane.

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[a]) This study was presented in part at the 37th Conference of the American Society for Mass Spectrometry and Allied Topics, May, 1989, Miami Beach, FL.

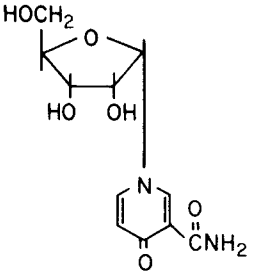
[b]) Deceased.

INTRODUCTION

For a number of years, our laboratory has been engaged in the isolation and characterization of novel substances excreted in the urine of cancer patients and normal subjects[1]. Of special interest have been the nucleosides and bases derived from catabolic and anabolic processes[2] and their potential use as indicators of aberrant cellular activity[3]. Certain of these modified nucleosides, such as N^2-Dimethylguanosine, derived from tRNA catabolism, are excreted in elevated quantities in the urines of patients with specific solid tumors and these levels have been correlated to therapy response[4]. In other malignancies, such as lymphoma, lower than normal values were reported[5]. Studies in this area[2] have demonstrated that a number of modified nucleosides, derived from tRNA turnover, can serve as useful markers in the diagnosis of certain cancers, in patient management and in providing a better understanding of the malignancy. Extension of these studies to immunodeficiency disorders[6] including AIDS[7] have proven to be of similar value.

Recently, our laboratory has characterized four modified novel nucleosides[8,9,10] isolated from the urine of CML patients but originating from sources other than tRNA. The origin of 7-β-D-Ribofuranosylhypoxanthine[9], 5'-Deoxy-5'-methylthioadenosine sulfoxide and 5'-Deoxyinosine[10] are unknown. Vitamin B_{12} and the two mammalian enzymes mediated by this vitamin suggest a possible source and invite speculation for a relationship between the abnormal serum Vit B_{12} levels recognized in patients with myeloproliferative diseases and the presence of these metabolites in their urine. The fourth nucleoside, 1-β-D-Ribofuranosyl-4-pyridone-3-Carboxamide (β-4-PCNR)[8], is a pyridone riboside structurally related to and presumably originating from NAD, or the free base nicotinamide, although neither its origin nor function has been established conclusively or clearly. The biochemical pathways which define the origins of these four nucleosides remain speculative. The finding of these urinary metabolites may, however, provide a stimulus for exploring new metabolic processes, thereby expanding our understanding of cellular biochemistry and its dysfunction in neoplasia.

In continuation of these studies, we wish to report the isolation and characterization of an anomer of β-4-PCNR identified as 1-α-D-Ribofuranosylpyridin-4-one-3-carboxamide (α-4-PCNR) (I) by UV, NMR, CD and GC/MS spectra and chromatographic mobility comparisons with synthetic samples of the α and β isomers. To our knowledge this is the first reported characterization of an α nucleoside found in urine which was isolated from 24 hour urine collections of one normal human subject (190μg) and 2 CML patients (400μg,460μg). The β-4-PCNR was isolated similarly (600μg) establishing a heterogeneity between the anomers and confirming the presence of both in the same normal urine.



(I)

1-α-D-ribofuranosylpyridin-4-one-
3-carboxamide (α-4-PCNR)

MATERIALS AND METHODS

Neutral charcoal (Norit) was purchased from Fisher Scientific Co. and Celite-545 was obtained from Johns-Mansville Co. DEAE cellulose (DE-23) and AG1-X8 formate (2000-4000 mesh) anion-exchange resin were obtained from Whatman and Bio-Rad Labs., respectively. Deuteurium oxide (99.96 atom % D) was purchased from Aldrich Chemical Co. Glass distilled methanol was obtained from Burdick and Jackson while the deionized distilled water used in RP-HPLC was prepared in our laboratory. AFFI-GEL 601 affinity gel was purchased from Bio-Rad Labs. Silylating agent bis(trimethylsilyl)trifluoroacetamide containing 0.1% trimethyl-chlorosilane and pyridine were purchased from Regis Chemical Co. An authentic sample of β-4-PCNR was prepared according to the reported procedure[8].

Utraviolet spectrophotometry (UV). Utraviolet spectra were recorded on a Cary 219 spectrophotometer which was zeroed with water using the auto baseline feature.

Nuclear magnetic resonance (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°C with the BVT-2000 temperature controller of the WP-200 spectrometer. The chemical shifts reported are given in (δ) ppm, and measured from internal TSP (sodium-3-trimethylsilylpropionate-2,2,3,3-d4). The urinary unknowns were lyophilized three times from 99.5% D_2O and then dissolved in 99.9% D_2O for NMR analysis.

Gas chromatography/mass spectrometry (GC/MS). Low resolution mass spectral studies and GC/MS studies were carried out using a Finnigan 4000 quadruple instrument interfaced to an INCOS data system.

All samples were analyzed as their trimethylsilyl (TMS) derivatives which were formed by heating approximately 0.02 A_{263} units (800 ng) of

vacuum dried material with anhydrous pyridine and bis(trimethylsilyl)-trifluoacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (1:1, 4µL) in a sealed melting point capillary tube at 78.5°C for 1 hour.

The fused silica capillary column (30 meter long, 0.25 mm i.d. 3% SE-30, 25µ film thickness) was interfaced directly into the ion source allowing for maximum sensitivity. Samples were injected in the splitless mode (injector temp. 280°C) and the column oven was temperature programmed from 80°C (2 min. hold) to 280°C at a rate of 10°C/min. for each run. All mass spectral information was obtained at an ionizing voltage of 70 eV.

Circular dichroism (CD). The CD studies were carried out on a Jasco Model 5 CD/ORD in neutral aqueous solutions (5.8×10^{-5}M).

Preparative reversed-phase high performance liquid chromatography (RP-HPLC). A reversed-phase Zorbax (Dupont) ODS C_{18} column (21.2 mm I.D. x 25 cm length, 8 µm) was used for preparative RP-HPLC. Other components of the preparative RP-HPLC system include: Rainin Rabbit HPX high pressure pump; Altex UV detector, Model 153, with a preparative flow cell set at wavelength 254 nm; Rheodyne sample injector fitted with a 5 ml loop, Model 7010, and gradient mixing bottles.

Semi-preparative RP-HPLC. The semi-preparative RP-HPLC was carried out on an Altex Model 332 gradient liquid chromatograph equipped with a system controller programmer, twin Altex 110A pumps, Rheodyne sample injector Model 7010, a LKB 2140 rapid spectral diode-array detector programmed with an AT&T PC 6300 equipped with WAVESCAN 2140-250 software for acquisition, storage and post-run evaluation of data, and a Canon PJ-1080A plotter. In some instances, an Altex Model 153 analytical UV detector with a 8 µL flow cell set at 254 nm and a C-R1A Altex integrator were used in place of the computerized diode-array detector. The purification and coinjection studies were performed on a Beckman reversed-phase Utrasphere ODS C_{18} semi-preparative column (10.0 mm I.D. x 25 cm length, 5µm) fitted with a 2 ml loop. The following RP-HPLC solvent systems (Table 2) were used for coinjection studies: A) isocratic, 10% methanol in water; B) gradient elution 0->25% methanol in 0.1M ammonium acetate buffer, pH 7.0, over 25 min.; C) gradient elution 0->20% methanol in water over 20 min.; D) isocratic, 6% methanol in 0.1M ammonium formate buffer, pH 4.3; E) gradient elution 0->10% methanol in 0.1M ammonium acetate buffer, pH 4.0, over 15 min.; F) isocratic, 5% methanol in water; G) isocratic, 15% methanol in water. All samples were run at a flow rate of 3 ml/min. at 25°C.

Isolation of Unknown Urinary Nucleoside (I) and β-4-PCNR. The unknown urinary nucleoside I and β-4-PCNR were isolated from a 24 hour urine collection (1500 ml) of a normal male human subject by the isolation protocol described previously[11]. The urinary cis-diols (containing the ribosides) were isolated in the 0.7M boric acid eluate from the DEAE-cellulose column. Initial fractionation was achieved by RP-

HPLC on a preparative Zorbax column with a gradient elution of 0->25% methanol in 0.1M ammonium acetate buffer, pH 7.0, in 1 hour, flow rate 8 ml/min., at 22°C (Fig 1).

The unknown I was isolated from a peak eluting at 24 min. and purified to homogeneity on a semi-preparative RP-HPLC column eluting at 10.8 min. with isocratic 10% methanol in water (190 µg). The β-4-PCNR was isolated and purified similarly, eluting at 28 min. from the Zorbax column and at 15.3 min. during final purification (600 µg). The unknown I was also isolated in the urine of 2 CML patients (400 µg, 460 µg).

Detection of the Unknown Urinary Nucleoside I and β-4-PCNR by boronate affinity gel chromatography. The coplanar cis-diol (functional group of the ribonucleosides) compounds were separated from the other urinary substances by a modification of a one-step boronate affinity gel micro chromatography procedure developed by Gehrke[12]. Our use of a larger column bed (1.0 x 9.0 cm) enabled us to process a 25 ml aliquot of urine. The column packing, urine preparation and chromatographic procedure were virtually identical to those reported by Gehrke except an increased buffer volume was necessary for the scaled-up procedure. Consequently, 15 ml of 2.5 M ammonium acetate buffer (pH 9.5) was added to the 25 ml urine sample before it was centrifuged and applied to the column. The resin bound cis-diol boronate complexed material was freed of other urinary impurities by washing with 40 ml of 0.25 M ammonium acetate buffer (pH 8.8) and 50 ml of 2.25 M ammonium acetate buffer (pH 8.8). The ribosides were eluted from the column with 150 ml of 0.1 N formic acid at which time the absorbance at 260 nm was less than 0.01. This cis-diol fraction (pH 4.5) was concentrated and injected onto an Utrasphere ODS C_{18} RP-HPLC column (1.0 x 25 cm x 5µm) where it resolved into more than 30 peaks. The eluting buffer was a gradient of 0->30% methanol in 0.01 M ammonium acetate buffer pH 4.4 over 60 min. at a flow rate of 3 ml/min. Peaks eluting at the retention times corresponding to the predetermined elution position of the selected standards (retention time ± 5 min.) were collected individually and the UV absorption spectra were obtained. These selected peaks were injected individually or coinjected with the appropriate standard and rechromatographed on the semi-preparative ODS C_{18} RP-HPLC column in several buffer systems (Table 2). The collective mobility characteristics, the on-line spectra obtained with the diode-array detector and the purity parameters of the WAVESCAN software confirmed or excluded homogeneity between the natural urinary material and the synthetic standard. The predetermined eluting positions of the 2 synthetic standards, α-4-PCNR and β-4-PCNR, were obtained by adding the known standard to an aliquot of the riboside eluate and chromatographing the "spiked" aliquot in an identical RP-HPLC system. Comparison of the retention times and increased peak areas with an "unaltered" urinary chromatogram identified the peaks of interest.

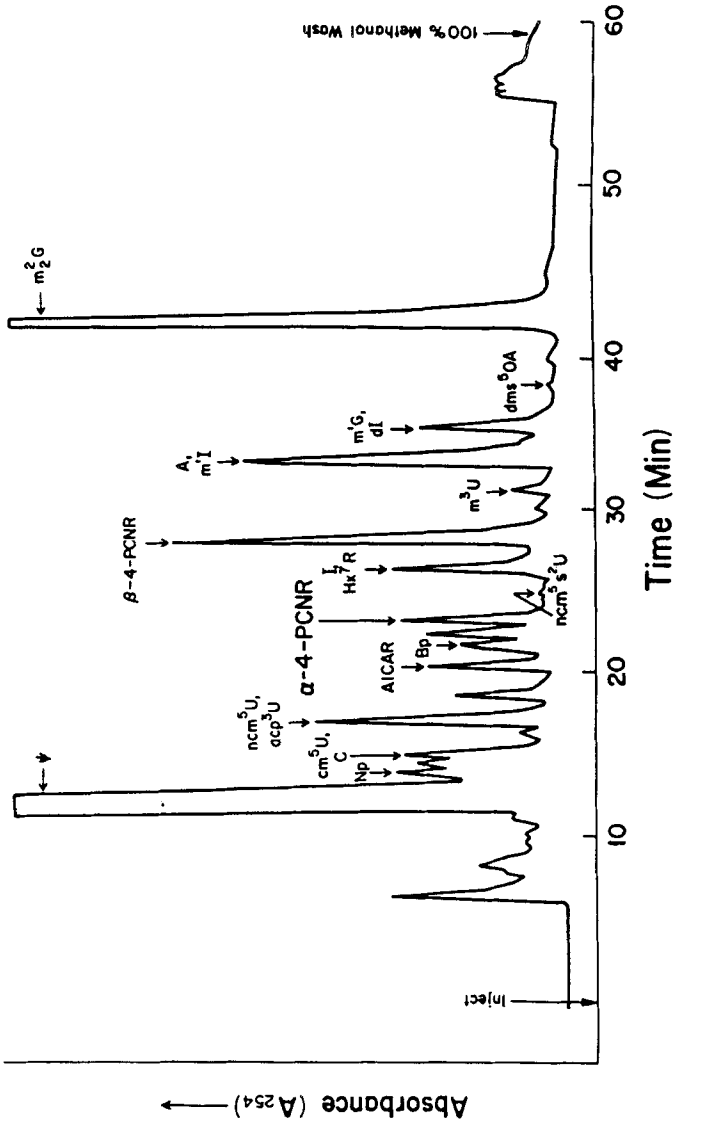
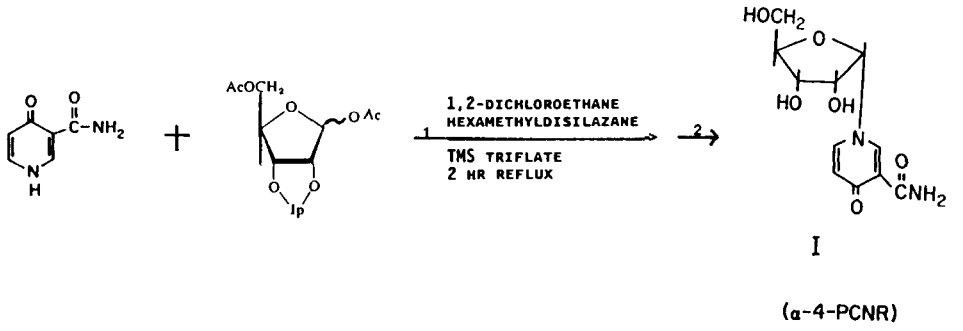


FIG. 1 Initial HPLC fractionation of the urinary cis-diol fraction on a Preparative Zorbax Column.

SYNTHESIS OF α-PCNR



I

(α-4-PCNR)

Scheme 1

Synthesis of α-4-PCNR (Scheme 1)[12a]. To 20 mg (0.145 mmol.) of 3-Carboxamido-4-pyridone[13] was added HMDS (5 ml) + TMCS (1 ml) and the reaction mixture was refluxed for 2 hours. The clear solution was evaporated to dryness and 2,3-isopropylidene-1,5-diacetyl β-D-ribofuranose (300 mg; 1.09 mmol) was added to it followed by freshly distilled 1,2-dichloroethane (5.0 ml) and a solution of TMS triflate in 1,2-Dichloroethane(1:4; 0.5 ml) and refluxed for 1 hour. To the brown solution was added MeOH (1 ml) and the mixture was warmed for 5 minutes and then evaporated to dryness. The residue was dissolved in NH_3-MeOH (4N; 10 ml) and stirred at room temperature overnight. The clear solution was evaporated to dryness and dissolved in 75% acetic acid in water (2 ml) and warmed on a steam bath for 4 hours. The solution was evaporated to dryness and run on an HPLC system using a C_{18} reverse phase (SP-18, 25 cm long; i.d. 1 cm) column with a gradient of 0-25% MeOH in water during 1 hour (8 ml/min). The major peak from the chromatography was collected and evaporated to dryness. UV λ_{max} at 259 (H_2O), 255 (0.1 N HCl) and 260 nm (0.1 N NaOH); yield ~1700 A_{259} units.

On GC/MS studies (3% SE 30 capillary column) the fraction showed one major peak (M^+ at m/z 558) which presumably was due to 3-carboxamido-4-pyridone-α-riboside (α-4-PCNR) and a trace peak (M^+ at m/z 558) which was identified as 3-carboxamido-4-pyridone-β-riboside (β-4-PCNR) by comparing the mass spectra and GC retention times with those of authentic standards. This fraction was then rechromatographed on C_{18} reversed phase (SP 18, 25 cm long; i.d. 1 cm) column and eluted isocratically with 10% MeOH in water for 1 hour (8 ml/min). Two fractions were collected.

Fraction #1. UV λ_{max} at 260 (H_2O), 256 (0.1 N HCl) and 259 nm (0.1 N NaOH); yield ~65 A_{260} units. This was identified as 3-carboxamido-4-

pyridone-β-riboside (β-4-PCNR) by GC/MS comparisons and TLC comparisons with an authentic sample.

Fraction #2. UV λ_{max} (Fig. 2) 259 (H_2O), 254 (0.1 N HCl) and 259 nm (0.1 N NaOH). This was identified as 3-carboxamido-4-pyridone-α-riboside (I) by GC/MS and TLC studies. This was then evaporated to dryness, dissolved in water (5 ml), filtered, lyophilized (yield 25.4 mg; 64.96%), and the lyophilized material dried under high vacuum over P_2O_5 at 60° for 8 hours; m.p. 105-108° (softens); 130-135° (melts). Anal. Calc. for $C_{11}H_{10}N_2O_6.5H_2O$: C, 47.31%; H, 5.37%; N, 10.04%; Found : C, 47.44%; H, 5.21%; N, 9.68%. The GC/MS spectrum (Fig. 3) of the trimethylsilylated compound showed a molecular ion at m/z 558 corresponding to a tetratrimethylsilyl derivative of I. The EI mass spectrum (Fig. 4) of the underivatized I showed a molecular ion at m/z 270. NMR, δ 8.72 (d, C_2-H, 1 H, $J_{2,6}$ 2.1), pair of doublets at 8.02 and 7.98 (C_6-H, 1 H, $J_{6,2}$ 2.3, $J_{6,5}$ 2.3), 6.75 (d, C_5-H, 1 H, $J_{5,6}$ 7.6) 6.05 (d, 1'-H, 1 H, $J_{1,2}$ 4.7) and a number of peaks in the region 5.0-3.5 ppm (2', 3' and 5'-protons of ribose (Table 1).

Stability of β-4-PCNR. A solution of synthetic β-4-PCNR containing 36 A_{260} units in 6 ml of saline was incubated at 25°C at pH 2.9 for 5 days. An aliquot was chromatographed on RP-HPLC in solvents A and F and coinjected with synthetic β-4-PCNR and α-4-PCNR to confirm product identity. Two mls of the 5 day solution were applied to an AFFI-GEL boronate affinity column and chromatographed as described previously. The riboside eluate and combined washes were examined by RP-HPLC. Three mls of the 5 day solution were subjected to a mock isolation procedure originally used to isolate (I) from the urine but with a scaled-down procedure. Disposable glass Pasteur pipettes were used as columns. The product in each eluate was analyzed and identified by RP-HPLC and the final product was identified by RP-HPLC and NMR. An aliquot of the standard solution (pH 2.9) was reexamined after 7 days by RP-HPLC. A portion of the standard solution mixture, incubated for 5 days at pH 2.9, was adjusted to pH 1.0 and incubated at the lower pH for 24 hours at 24°C and then examined by RP-HPLC. Products, in all instances, were identified by their co-migration properties with appropriate standards in discriminating mobile-phase solvents (Table 2) and by on-line full UV spectra obtained with the diode-array detector on the RP-HPLC. In some instances, homogeneity between product and identifying standard were confirmed through the purity parameter software of WAVESCAN.

RESULTS

The UV spectra of unknown urinary nucleoside I exhibited a λ_{max} at 257 nm at pH 1.3, 258 nm at pH 6.0 and 259 nm at pH 11.0 (Fig. 2a). It was very similar to the UV spectra of β-4-PCNR isolated from the urine of a CML patient, and characterized in our laboratory previously[8]. The

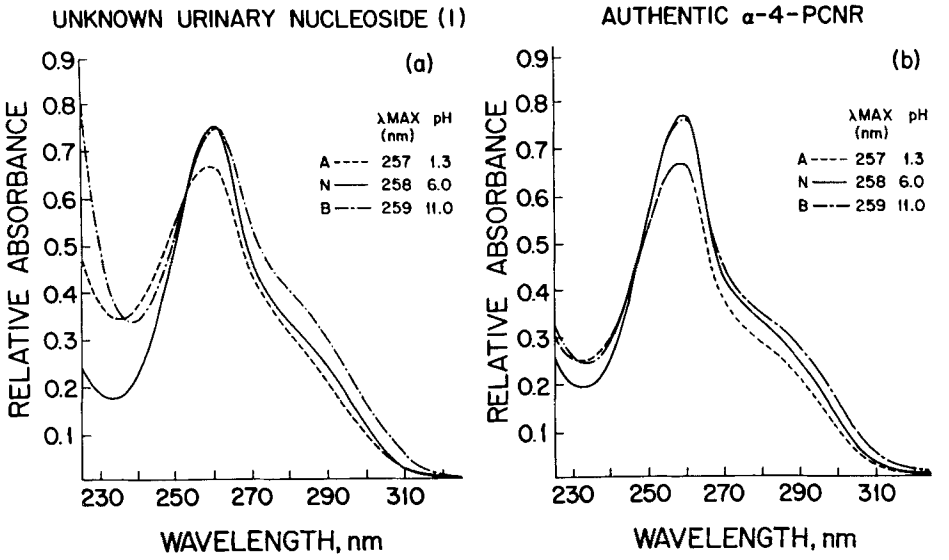


FIG.2 Ultraviolet absorption spectra of synthetic α-4-PCNR and the unknown urinary nucleoside (I).

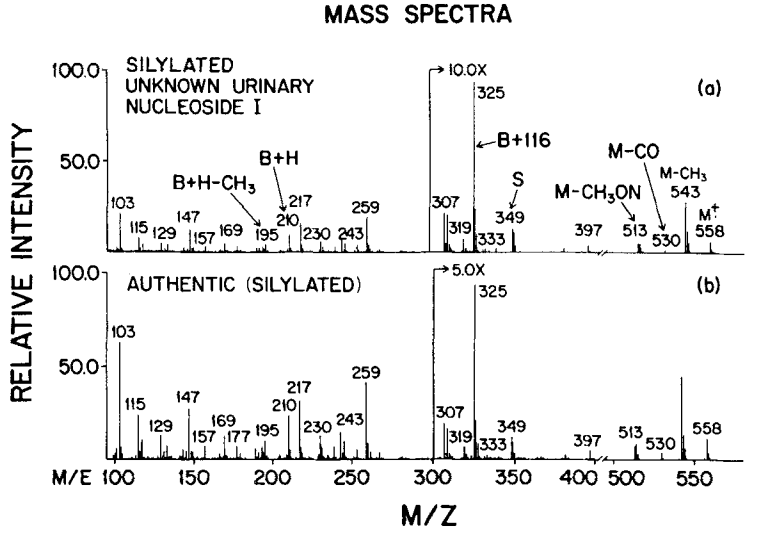


FIG. 3 GC/MS spectra of tetratrimethylsilyl derivative of synthetic α-4-PCNR and unknown urinary nucleoside (I).

recognized β-4-PCNR was isolated during the current preparative RP-HPLC eluting 4 min. after I. A 1 nm blue shift of the λ_{max} in the UV spectra of I when compared to the UV λ_{max} of authentic β-4-PCNR at neutral pH and retention time differences in several buffers on RP-HPLC affirmed the distinction between the two nucleosides.

The GC/MS spectra of trimethylsilyl derivative of I (Fig. 3a) exhibited the highest mass ion at m/z 558 along with other characteristic fragment ions identified in the mass spectrum of the tetratrimethylsilyl derivative of β-4-PCNR[8]. These included a $(M-15)^+$ ion at m/z 543, a $(M-28)^+$ ion at m/z 530 attributed to the loss of CO, the unusual ion at m/z 513 indicative of a loss of CH_3NO and the ion at m/z 349 resulting from the sugar portion of I. These data suggest that I is an isomer of β-4-PCNR with an identical underivatized Mol. wt. of 270. The mass ions at m/z 349, 259, 245, 243, 230, 217 and 169 characteristic of unmodified ribose[14] preclude a substitution on the sugar. The isolation of I in the cis-glycol fraction from a borate-cellulose column supports a ribose sugar.

The EI mass spectra of underivatized I has the highest mass ion at m/z 270 confirming a Mol. wt. of 270 and fragment ions consistent with a ribose nucleoside (Fig. 4). These include the base ions at m/z 138 $(b + H)^+$ and 167 $(b + 30)$[14,15,16]. Ions at m/z 121 and 150, formed by the elimination of 17 amu, most likely represent a loss of NH_3. The loss of 44 amu represents the elimination of $CONH_2$ and leads to an ion at m/z 93 $(b-CONH_2)^+$. The ion at m/z 133 indicates an unmodified ribose. High resolution FAB spectrum of the natural unknown I in glycerol matrix gave a $(M+H)^+$ ion at 271.0931 confirming the molecular formula to be $C_{11}H_{14}N_2O_6$.

The NMR spectra of I and β-4-PCNR in D_2O (Table 1) confirm the isomeric distinctions between the two compounds and suggest I as having an α- configuration. The proton splitting patterns and chemical shifts for both anomers are similar but not identical (Table 1). The doublets at 6.05 ppm for I and 5.71 ppm for β-4-PCNR are resonances attributed to the anomeric protons and are presumptively indicative of α and β isomers[9,17]. The resonance signals of the ribose protons are markedly dissimilar, substantiating a configurational difference at the anomeric site. It should be mentioned that the two structural isomers 1-β-D-ribofuranosyl-2-Pyridone-3-Carboxamide and 1-β-D-ribofuranosyl-2-pyridone-5-carboxamide were excluded by their UV absorption spectra[18]. All the data reported so far is consistent with identifying the unknown urinary nucleoside I as the α anomer of β-4-PCNR (1-α-D-ribofuranosyl-4-Pyridone-3-Carboxamide).

The authentic α-4-PCNR was, therefore, synthesized for comparison studies (Scheme 1) and in all physico-chemical comparisons I proved identical to the synthetic α-4-PCNR. The UV spectra were identical in acid, base and neutral media (Fig. 2a,b) as were the GC/MS spectra of the

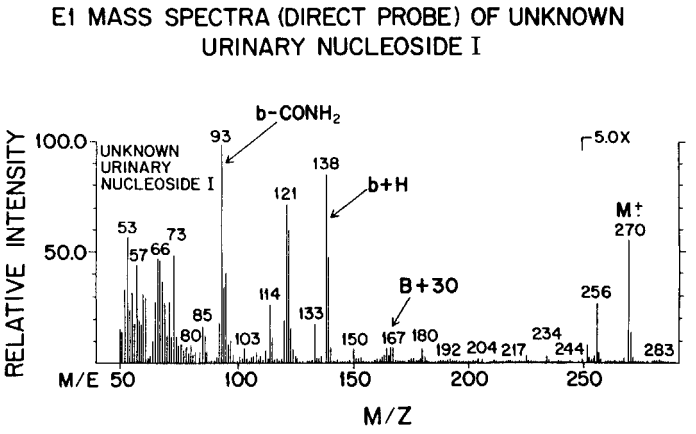


FIG. 4 EI mass spectrum of unknown urinary nucleoside (I).

TABLE 1

CHEMICAL SHIFTS (δ) AND J VALUES (Hz) FOR UNKNOWN URINARY NUCLEOSIDE (I) AND AUTHENTIC α-4-PCNR AND β-4-PCNR IN D_2O

COMPOUNDS	$C_{2H}(J_{2,6})$	$C_{6H}(J_{6,2})$	$C_{6H}(J_{6,5})$	$C_{5H}(J_{5,6})$	$C_{1'H}(J_{1',2'})$
UNKNOWN URINARY NUCLEOSIDE (I)	8.72(2.1)	8.02(2.3)	7.98(2.3)	6.75(7.6)	6.05(4.7)
AUTHENTIC α-4-PCNR	8.72(2.0)	8.02(2.2)	7.98(2.1)	6.75(7.7)	6.05(4.6)
AUTHENTIC β-4-PCNR	8.86(2.3)	8.13(2.2)	8.09(2.0)	6.79(7.6)	5.71(5.2)

tetratrimethylsilyl derivatives (Fig.3a,b). The NMR spectra in D_2O were also in complete agreement (Table 1 and Fig. 5).

The CD spectra of synthetic α-4-PCNR exhibited a negative band in aqueous solution with a λ_{max} at 254 nm. It was superimposable on the CD spectra of I and readily distinguished from the CD spectra of β-4-PCNR.

Final identification was by RP-HPLC coinjection studies. A mixture of natural and synthetic α-4-PCNR co-migrated as a single discrete peak with identical mobility characteristics in 5 solvent systems (Table 2).

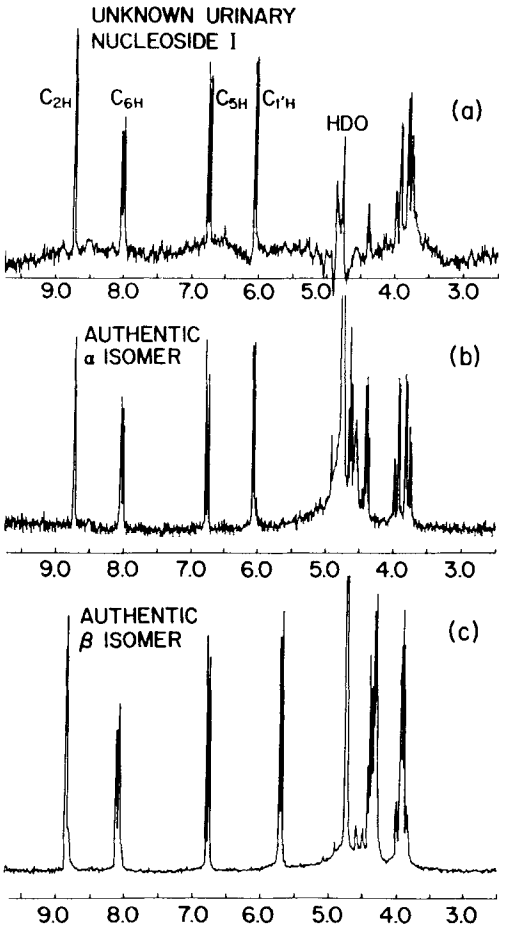


FIG. 5 NMR spectra of authentic β-4-PCNR (c), α-4-PCNR (b) and unknown urinary nucleoside (I).

A mixture of synthetic β-4-PCNR and natural I resolved into two peaks. These studies unequivocally identified I as the α-4-PCNR (Table 2).

Having assigned a structure to I, our attention was directed to answering the obvious question. Is β-4-PCNR stable under the conditions encountered during the processing and isolation of I or does anomerization occur generating the α anomer during the process of isolation? It is known that structurally similar β-NADH is acid labile, converting to an α-nicotinamide decomposition product at pH 3.5 through anomerization of the pyridine-ribose bond[19,20]. The urine was subjected to this pH during processing. In an attempt to answer this question, a solution of

TABLE 2

COMPARISON OF RP-HPLC RETENTION TIME (MIN) OF UNKNOWN URINARY NUCLEOSIDE I AND AUTHENTIC α-4-PCNR AND β-4-PCNR

	SOLVENT SYSTEM*						
COMPOUND	A	B	C	D	E	F	G
UNKNOWN URINARY NUCLEOSIDE (I)	8.82	18.84	17.03	14.76	18.43	17.00	7.12
AUTHENTIC α-4-PCNR	8.78	18.73	17.17	14.88	18.42		
MIXTURE OF (I) AND AUTHENTIC α-4-PCNR	8.80	18.55	17.00	14.83	18.33		
AUTHENTIC β-4-PCNR	11.30		20.13			28.0	8.68
COINJECTED MIXTURE OF (I) AND AUTHENTIC	8.70					17.02	7.12
β-4-PCNR	11.40					28.19	8.87

*See Materials and Methods

synthetic β-4-PCNR was acidified to pH 2.9 at ambient temperature. After 5 days the solution was shown to contain only the original β-4-PCNR when chromatographed and analyzed by RP-HPLC. After 7 days, a trace of degraded product was detected by RP-HPLC but its UV absorbance (λ_{max} at 280 nm) was unlike that of α or β-4-PCNR (λ_{max} at 258). It was more hydrophilic (r.t. 6.05 min.) than the α (r.t. 9.0 min.) or β (r.t. 12.0 min.) anomers in 10% aqueous methanol mobile phase. Its failure to complex on a boronate affinity gel column indicates the loss of the cis-glycol functionality of ribose and suggests the free base. A sample acidified to pH 1.0 decomposed to the extent of about 70-80% to a new product (λ_{max} 280 nm) in 24 hours. In neither sample was α-4-PCNR detected. Clearly β-4-PCNR decomposes on prolonged and severe acidification with no evidence of anomerization to the α product.

To further ensure that conversion of β-4-PCNR to the α anomer had not occurred during our isolation procedure, an aliquot of acidified synthetic β-4-PCNR was subjected to a control purification procedure similar to that used in isolating natural I. RP-HPLC and NMR identified original β-4-PCNR as the sole detectable product isolated from the entire fractionation procedure. There was no detectable evidence of α-4-PCNR or any other transformed product in any of the eluates.

Finally, to affirm the presence of both anomers in the same urine sample and exclude, more convincingly, the possible formation of I during our isolation process, a milder one-step boronate affinity gel procedure developed by Gehrke[12] to segregate and identify the cis-diol compounds from urine was initiated. The urinary ribose fraction, segregated on the boronate affinity gel column, was concentrated and fractionated on a semi-preparative RP-HPLC column. The peaks eluting with retention times similar to those of the two synthetic standards, α-4-PCNR (19.0 min) and β-4-PCNR (23.0 min), were collected individually, and rechromatographed on RP-HPLC. Appropriate identification was established by comparison of on-line spectra obtained with a diode-array detector and migration similarities with the identifying synthetic standards in 4 discriminating solvent systems (Table 2). The two isomers were detected in the urines of the 2 normal human subjects and a CML patient examined by this protocol. The acidified β-4-PCNR standard, chromatographed similarly, was stable to this isolation scheme with no evidence of anomerization to the α-4-PCNR.

It can be concluded, therefore, that both the α-4-PCNR I and β-4-PCNR are present in urine and I does not appear to originate from β-4-PCNR during isolation.

DISCUSSION

The structure of I has been assigned as 1-α-D-Ribofuranosylpyridin-4-one-3-carboxamide (α-4-PCNR) based on UV, NMR, GC/MS and CD spectral data and chromatographic behavior on HPLC. Absolute confirmation of the structure was obtained by chemically synthesizing this compound and demonstrating that the natural and synthetic materials were identical. Clearly α-4-PCNR is structurally related to nicotinamide adenine dinucleotide (NAD) and the free base nicotinamide. It is well established, that the major urinary metabolites of nicotinamide and nicotinic acid in man are the 2- and 4- oxo derivatives of 1-methyl-nicotinamide[10b,21]. The 1-methylpyridin-2-one-5-carboxamide has been identified as the primary human catabolite[22]. Excreted in lesser amounts is 1-methylpyridin-4-one-3-carboxamide[23] while a third isomer, 1-methylpyridin-2-one-3-carboxamide[24], is not excreted in appreciable amounts in human urine. Recently, the ribosides of two of these metabolites, 1-β-D-Ribofuranosylpyridin-2-one-5-carboxamide (β-2-PCNR)[18]

and 1-β-D-Ribofuranosylpyridin-4-one-3-carboxamide (β-4-PCNR)[8] have been isolated from human urine. While their origin as catabolic endproducts has been suggested[25], a direct metabolic pathway has not been demonstrated. Reported here is the isolation and identification from human urine of the α anomer I of β-4-PCNR (α-4-PCNR).

The origin of α-4-PCNR is unknown and open to speculation. One obvious concern was the possible formation of I from β-4-PCNR during the isolation and purification process. It was especially critical to establish that β-4-PCNR did not anomerize on acidification as the related β-NADH is known to convert to an α-nicotinamide derivative at lower pH. The identification of I in urine by an alternate milder procedure and the demonstrated stability of acidified β-4-PCNR in both the original and the second isolation procedures argues against this *in-vitro* anomerization. The finding of I in 3 urines that were collected and handled under strictly controlled conditions, indicates post-excretion formation as highly improbable. The rationale that suggests β-4-PCNR and β-2-PCNR represent metabolites of β-NAD may be extended to include a metabolic origin of α-4-PCNR from α-NAD (Fig 6).

Unquestionably, the pyridones are recognized catabolites of nicotinamide, the free base of the natural cofactor β-NAD. The possibility of catabolic origin of α-PCNR from natural α-NAD, therefore, deserves attention. Isolation of α-NAD from numerous sources, including animal tissue, has been reported. These reports have led to speculations concerning its *in-vivo* origin and its role in metabolic functions[24a]. Proposals regarding biological functions of α-NADH have also been reported[26]. The validity of these findings have been questioned and an artifactual origin of α-NAD and α-NADH, promoted by conditions encountered during isolation, have been suggested[27]. It has been shown, however, that at physiological pH, β-NADH in the presence of oxygen and excess hydrogen ions anomerizes to α-NAD[28]. Although the biological occurrence and significance of the α-anomer is unclear, the possible metabolic origin of α-PCNR from a natural α-NAD precursor remains attractive.

Microorganisms constitute a primary source of naturally occurring α nucleosides. Dietary intake or *in-vivo* formation by intestinal flora, therefore, may account for the presence of I in the urine. A pathological source is excluded, however, as I was found in the urine of a healthy male volunteer.

Implying any functional importance for I would be premature at this time, but a number of nucleosides having a α-glycosyl linkage between the base and sugar have been shown to be biologically active. Certainly, several synthetic α nucleoside analogs have proven pharmacologically valuable as antitumor agents[29], bacteriostatic agents[30] and cytostatically active agents[31,32]. In enzyme studies, selective inhibitory action on mammalian DNA polymerase has been noted[31]. In at least one instance, a

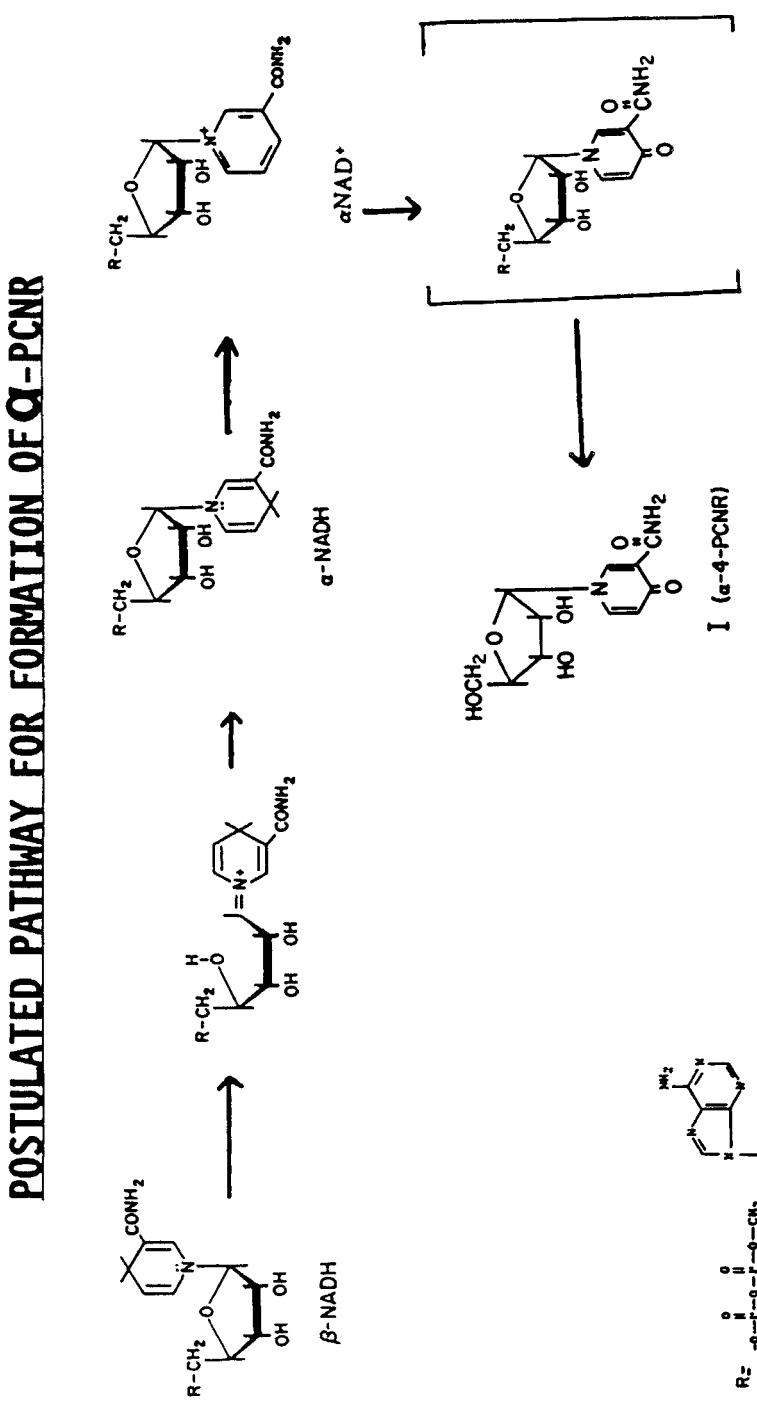


FIG. 6

biologically active molecule essential for human metabolism has an α ribose as part of its structure. Cobalamin (Vit. B_{12}) contains an α linkage between the 5:6 dimethylbenzimidazole and D-ribose, but natural analogs containing purines in place of the benzimidazole ring have been isolated[33]. The α configuration in Vit. B_{12} is essential for full expression of its full activity as a cofactor in the two human enzyme systems mediated by this vitamin[10]. Some similar role or one of agonist or antagonist in some metabolic pathway cannot be excluded for I. Oppenheimer[34] has demonstrated that the oxidation of α-NADH to α-NAD occurs with a stereochemistry of oxidation identical to that found with β-NADH by four diverse dehydrogenases from bacterial, yeast and mammalian sources. Other α derivatives may, therefore, possess similar unrecognized biological properties. It has yet to be demonstrated that α-4-PCNR is relevant to any physiological function but its significance as a urinary metabolite deserves further investigation.

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