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Structure determination of two new amino acid-containing derivatives of adenosine from tRNA of thermophilic bacteria and archaea

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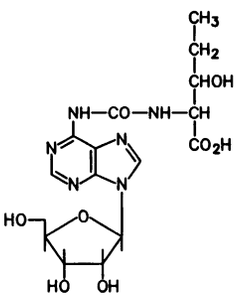
ABSTRACT

Two new nucleosides have been identified in unfractionated transfer RNA of two thermophilic bacteria, *Thermodesulfobacterium commune*, and *Thermotoga maritima*, six hyperthermophlic archaea, including *Pyrobaculum islandicum*, *Pyrococcus furiosus* and *Thermococcus* sp. and two mesophilic archaea, *Methanococcus vannielii* and *Methanolobus tindarius*. Structures were determined primarily by mass spectrometry, as 3-hydroxy-*N*-[[(9-β-D-ribofuranosyl-9*H*-purin-6-yl)amino]carbonyl]norvaline, (hn^6A), structure 1, and 3-hydroxy-*N*-[[(9-β-D-ribofuranosyl-9*H*-2-methylthiopurin-6-yl)amino]carbonyl]norvaline (ms^2hn^6A), 2. The amino acid side chain was characterized as 3-hydroxynorvaline (3) by gas chromatography-mass spectrometry of the trimethylsilyl derivative after cleavage from 1 and 2 by alkaline hydrolysis. Evidence for the amino acid-purine carbamoyl linkage was obtained from the collision-induced dissociation mass spectrum of trimethylsilylated 1, and the total structure was confirmed by chemical synthesis of 1.

INTRODUCTION

The posttranscriptional processing of tRNA has been shown to result in some modifications which are highly characteristic of phylogenetic placement among the three primary domains (eukarya, bacteria, archaea (1)), both with respect to sequence location of modified residues (2,3), and to molecular structure at the nucleoside level (4,5). Such modifications are also apparent in the tRNA of thermophilic bacteria, in which they are influenced by the temperature of cell growth (6,7), and exert a pronounced effect on tRNA melting temperature (8–10). In the case of archaea (archaebacteria), which include the highest temperature known organisms (optimal growth to 105°C (11)), a number of new modified tRNA nucleosides have been discovered and characterized (12–14), some of which are unique to the high temperature organisms (15) and which have been shown to confer conformational stability to individual nucleoside residues (16). Although little is known concerning stabilization of tRNA tertiary structure in archaeal hyperthermophiles, it is clear from phylogenetic patterns of modification (5,15) that bacteria and archaea have to a large extent developed independent mechanisms of posttranscriptional modification which may contribute, in parallel to G-C content (8), to thermal stabilization of tRNA.

We presently report the structural characterization of two nucleosides, compounds **1** and **2**, discovered in unfractionated



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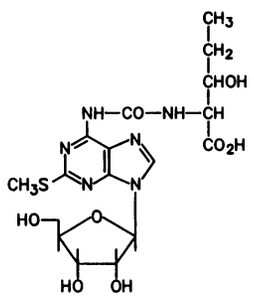

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tRNA from two thermophilic bacteria, *Thermodesulfobacterium commune* and *Thermotoga maritima*, and from six thermophilic and two non-thermophilic archaea. Both **1** (initially designated N27.4 from its HPLC retention time) and **2** (N31.3) contain the

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amino acid 3-hydroxynorvaline covalently bound through a carbonyl bridge to N^6 of adenosine. The structures were established principally from tRNA isolated from *T. commune*, based on mass spectrometry, and also from chemical synthesis of **1**, to conclusively establish the position and nature of the amino acid-base linkage.

EXPERIMENTAL

Cells

T. commune was grown at 70°C in a 30 L batch in an anaerobic fermentor under N_2 and (towards the end of growth) under N_2/CO_2 (90:10 v/v). The medium was a Na_2SO_4-containing fresh water medium (low NaCl and $MgCl_2$) (17), with elevated KH_2PO_4 concentration (0.6 g/L) but without $NaHCO_3$ and Na_2S. Before innoculation from a 1.6 L batch, the O_2-free, N_2-sparged medium was reduced with fresh, anoxically-prepared $Na_2S_2O_4$ (15 mg/L final concentration). The organic substrate was 40 mM pyruvate added from a filter-sterilized stock solution; the latter was prepared from a 28.7% (vol/vol) pyruvic acid solution in water by dropwise addition of the same volume of a 4.0 M NaOH solution under stirring in an ice bath. At the end of the exponential phase, the batch was cooled and cells were harvested in a Sharple flow centrifuge.

Culture conditions for the following organisms were reported previously (15): *Methanolobus tindarius*, *Pyrobaculum islandicum*, *Pyrodictium occultum*, *Thermococcus* sp., *Thermodiscus maritimus*, *Thermoproteus neutrophilus*, and *Thermotoga maritima*. *Pyrococcus furiosus* tRNA was obtained from J.A. Kowalak, and had been isolated from cells (strain DSM 3638) grown at 100°C; details will be reported separately (18). *Methanococcus vannielii* tRNA was a gift from T. Stadtman.

Isolation of transfer RNA

Transfer RNA was extracted from *T. commune* cells using a previously-described protocol (19). For final purification of the 2 M LiCl-soluble tRNA fraction, DEAE-cellulose column chromatography (19) was replaced by clean-up on a Nucleobond AX-500 cartridge (Nest Group, Southborough, MA). Transfer RNA was eluted with 0.65 M KCl in 100 mM Tris-phosphate (pH 6.3)/15% EtOH buffer (equal volumes of manufacturer's buffers N1 and N2). Transfer RNA was precipitated by addition of 0.8 vol isopropanol to the eluate which was placed on ice for 30 minutes, and recovered by centrifugation. One cartridge was used for multiple 2 mg loadings after re-equilibration with starting (N1) buffer. The pellet was dried *in vacuo* until used. Isolation of tRNA from other organisms studied was as previously described (15).

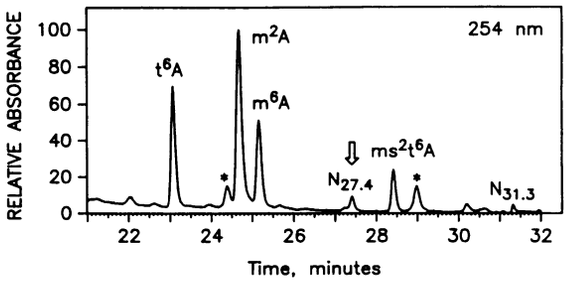


Figure 1. Chromatographic separation of nucleosides from HPLC of an enzymatic hydrolysate of unfractionated *Thermodesulfobacterium commune* tRNA, with UV detection at 254 nm. N27.4 and N31.3 are the two nucleosides reported in the present study; the asterisk denotes modified nucleosides not yet characterized. The arrow shows the elution time of synthetic D,L-3-hydroxy-*N*-[[(9-β-D-ribofuranosyl-9*H*-purin-6-yl)amino]carbonyl]norvaline measured in a separate experiment.

Enzymatic digestion of transfer RNA

For analytical-scale (50 μg) chromatographic analyses, tRNA was digested with nuclease P1, venom phosphodiesterase (VPD) and bacterial alkaline phosphatase (BAP) using the enzyme sources and protocols previously described (20). For preparative chromatography (3.5−5 mg scale), enzyme amounts were decreased 10-fold and the digestions (nuclease P1 and BAP) were allowed to proceed overnight at 37°C, with the intervening VPD digestion proceeding for 8 hr.

Directly-combined liquid chromatography/mass spectrometry (LC/MS) and preparative high-performance liquid chromatography (HPLC)

LC/MS analyses were performed as previously described (21) in which the HPLC effluent from reversed-phase HPLC (19,21) was passed through an intermediate UV detector, to a thermospray interface of a quadrupole mass spectrometer. Measurements were typically carried out on 25−50 μg of digested tRNA. Prior removal of enzymes is not necessary. Mass spectra in Figure 2 were acquired from an enzymatic digest of *T. neutrophilus* tRNA. Preparation of HPLC solvents using ^2H_2O (Merck Isotopes, St. Louis, MO) and LC/MS for determination of exchangeable hydrogen atoms in nucleoside mixtures has been described (21,22). Transfer RNA from frozen *T. commune* cells was used for preparative isolation of N27.4 and N31.3, which was effected on a Supelcosil™ SPLC-18-DB column (250 mm×10 mm i.d.; Supelco, Inc., Bellefonte PA) using an ammonium acetate/(aq) acetonitrile gradient as described (23), except that the ammonium acetate concentration was decreased to 0.1 M.

Trimethylsilylation of N27.4

One-fifth of the nucleoside recovered from 3 mg of *T. commune* tRNA was transferred into a 4 mm i.d. Pyrex capillary tube and dried in a Speed Vac (Savant Instruments, Farmingdale, NY). Ten μL of dry pyridine was added to the tube and removed *in vacuo* to further dry the nucleoside. Ten μL of dry pyridine was mixed with the contents of one 100 μL ampoule of Sylon BFT (*N,O*-bis(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane) (Supelco, Bellefonte, PA). Ten μL of this reagent was added to the dried nucleoside, and the tube was flame-sealed and allowed to stand for two hrs at room temperature and then for 30 min at 60°C.

Fast atom bombardment mass spectrometry (FAB-MS) and tandem mass spectrometry (FAB-MS/MS) of silylated nucleoside N27.4

Fast atom bombardment (FAB) mass spectra were acquired on a VG 70-SEQ instrument with a VG 11−250J data system (VG Analytical, Manchester, UK). One μL of 3-NO_2-benzyl alcohol matrix (Aldrich) was placed on the target and 1 μL of the N27.4 silylation reaction solution was added to the matrix droplet. The accelerating potential was 8 kV, and Xe atoms (7 kV) were used as the primary FAB beam. The same conditions were used for tandem mass spectrometry (MS/MS). Xe was used for the collision gas at a collision cell pressure sufficient to attenuate the precursor ion beam by 50%. Collision energy was 40 eV.

Alkaline hydrolysis of nucleosides N27.4 and N31.3

Conditions for microscale alkaline hydrolysis of N27.4 and N31.3 were adapted from earlier studies on t^6A (*N*-[[(9-β-D-ribofuranosyl-9*H*-purin-6-yl)amino]carbonyl]threonine) (24), using t^6A isolated by HPLC from *T. commune* tRNA. The collected N27.4 and N31.3 fractions (submicrogram amounts) obtained by preparative HPLC were each transferred to a reaction tube fashioned from 4 mm i.d. Pyrex tubing, and were dried under vacuum. Three μL of 0.1 N sodium hydroxide was carefully added to these dried samples without touching the walls of the tube with the syringe. The tube was sealed and heated for 4 hrs at 100°C to liberate the amino acid and nucleoside base. After cooling to room temperature the hydrolysate was neutralized with 10% acetic acid and dried under vacuum.

Trimethylsilylation of alkaline hydrolysates of N27.4 and N31.3

The dried reaction mixture was redried azeotropically using dichloromethane for removal of trace quantities of water. Trimethylsilylation (25) of the released amino acid was carried out by addition of 3 μL of a mixture of *N,O*-bis(trimethylsilyl)trifluoroacetamide and acetonitrile (1:1) directly to the tube in which hydrolysis was carried out and heating it at 100°C for 1 hr. This procedure also silylates the adenine base.

Gas chromatography/electron ionization mass spectrometry (GC/EI-MS)

All GC/EI-MS analyses were carried out on the VG 70-SEQ mass spectrometer equipped with a Hewlett Packard 5890 gas chromatograph. Gas chromatographic separation was performed on a DB-1 fused silica column (10 m×0.25 mm, film thickness 0.1 μm; J&W Scientific, Folsom, CA), the outlet of which was inserted directly into the ion source. Helium was used as the carrier gas at a column head pressure of 20 Kpa, resulting in a gas linear velocity of 40 cm/sec. Samples were injected in the splitless injection mode at an injection port temperature of 150°C. The oven temperature was initially set at 50°C, and was raised immediately after sample injection to 80°C at a rate of 15°C/min during solvent front passage, and then to 250°C at a rate of 10°C/min. Separator oven and the transfer line were maintained at 250°C and 260°C, respectively.

The mass spectrometer was operated in the electron ionization mode at an ion source temperature of 230°C and at 8 kV accelerating potential. The energy of the primary electrons was 70 eV. Mass spectra were acquired over the range *m/z* 50− 500 at a rate of 1.5 sec/decade with 0.6 sec interscan delay.

Synthesis of D,L-3-hydroxy-*N*-[[(9-β-D-ribofuranosyl-9*H*-purin-6-yl)amino]carbonyl]norvaline, (hn^6A)

Due to the unavailability of L-3-hydroxynorvaline, the D,L mixture was synthesized, using a method similar to that reported (26) for the synthesis of t^6A, but was simplified by eliminating isolation of the synthetic intermediate, crystalline ethyl-9-(2′,3′,5′-tri-*O*-acetyl-β-D-ribofuranosyl)-9*H*-purine-6-carbamate.

A solution of 2′,3′,5′-tri-*O*-acetyladenosine (39.3 mg, 0.1 mmol; Sigma) in anhydrous pyridine was cooled to −10°C in a dry ice/isopropanol bath. Ethyl chloroformate (29 μL, 33 mg, 0.3 mmol) was added drop-wise over a period of 20 min at approximately −10°C with stirring. The solution was allowed to warm to room temperature (23°C) and was stirred overnight. The resulting clear pale pink solution was evaporated to a dry residue which was co-evaporated twice with a small amount of toluene, and finally dissolved in chloroform (approximately 1.5 mL). The solution was washed with a saturated solution of sodium hydrogen carbonate followed by water. The chloroform layer was dried over anhydrous magnesium sulfate, and evaporated to give a pale yellow crystalline solid.

This product, mainly ethyl 9-(2′,3′,5′-tri-*O*-acetyl-β-ribofuranosyl)-9*H*-purine-6-carbamate, was dissolved in pyridine (0.5 mL), and was added to a suspension of DL-3-hydroxynorvaline (18.6 mg, 0.14 mmol; Sigma) in pyridine (0.5 mL). The reaction mixture was sealed and heated at 110°C for two hr and then 125°C for one hr, and was allowed to cool to room temperature. Pyridine was removed *in vacuo*. The brown residual solid was dissolved in methanol saturated with ammonia, maintained at 4°C overnight, then evaporated to dryness. The residue was dissolved in water and subjected to reversed-phase semi-preparative HPLC (Supelcosil SPLC-18-DB, 10×250 mm) using a 50 mM ammonium bicarbonate-acetonitrile (91.5:8.5) mobile phase pH 7.0, at 3 mL/min. The hn^6A-containing peak eluting at 21 min (retention time using the same gradient system as Figure 1 was 27.4 min) was collected and evaporated to dryness. Overall yield 12.2 mg (28.6%). FAB-MS (glycerol matrix): MH^+ 427; BH 295. UV, $\lambda_{max}^{H_2O}$ 268 nm; 275 nm (shoulder); lit for t^6A (27): $\lambda_{max}^{pH6.5}$ 270 nm; 277 nm (shoulder).

RESULTS

Analysis of unfractionated tRNAs by combined HPLC-mass spectrometry

LC/MS was used to establish the presence of nucleosides N27.4 and N31.3 in enzymatic hydrolysates of tRNA from ten organisms. The chromatogram shown in Figure 1 shows a representative example of the region of elution of components N27.4 and N31.3. Nucleoside assignments shown in Figure 1 are based on chromatographic retention times measured under standardized conditions, and on corresponding mass spectra measured every 1.6 sec during the chromatographic separation, which were then compared with cataloged data for RNA nucleosides (21,28). Neither the mass spectra (Figure 2) nor HPLC retention times of N27.4 and N31.3 corresponded to known constituents of RNA (21). The three principal ions marked in Figures 2A and 2B were determined as likely to belong to a single nucleoside in each case, from examination of time-based ion profiles for each mass value (21). The occurrences of nucleosides N27.4 and N31.3, established by LC/MS, are summarized in Table 1. Criteria for identification were retention times (internally referenced to a common component such as adenosine) of 27.4 ± 0.25 and 31.3 ± 0.25 minutes, associated with ion signals of *m/z* 134, and usually either 136 and 268, or 182 and 314.

Ions *m/z* 136 and 268 in Figure 2A represent the protonated adenine fragment ion (BH) and protonated adenosine molecular ion (MH^+) (28), respectively, and are characteristic of adenosine and its N-substituted derivatives (21). In Figure 2B these ions are shifted 46 mass units higher (to *m/z* 182, 314), consistent with methylthio substitution, for example as observed in the analogous mass spectrum of ms^2t^6A (*N*-[[(9-β-D-ribofuranosyl-9*H*-2-methylthiopurin-6-yl)amino]carbonyl]threonine) compared with t^6A (21). The *m/z* 134 ion, common to both nucleosides N27.4 and N31.3 (Figure 2) is judged to arise from a side chain, confirming that no molecular ions for N27.4

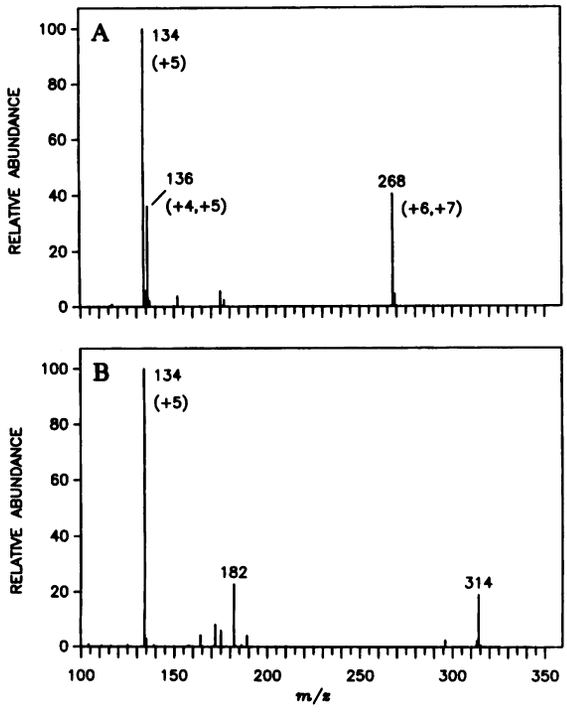


Figure 2. Thermospray mass spectra of nucleosides (**A**) N27.4 and (**B**) N31.3. Values in parentheses denote mass shifts resulting from hydrogen-deuterium exchange in separate experiments in which D_2O/ND_4OAc HPLC solvents were used.

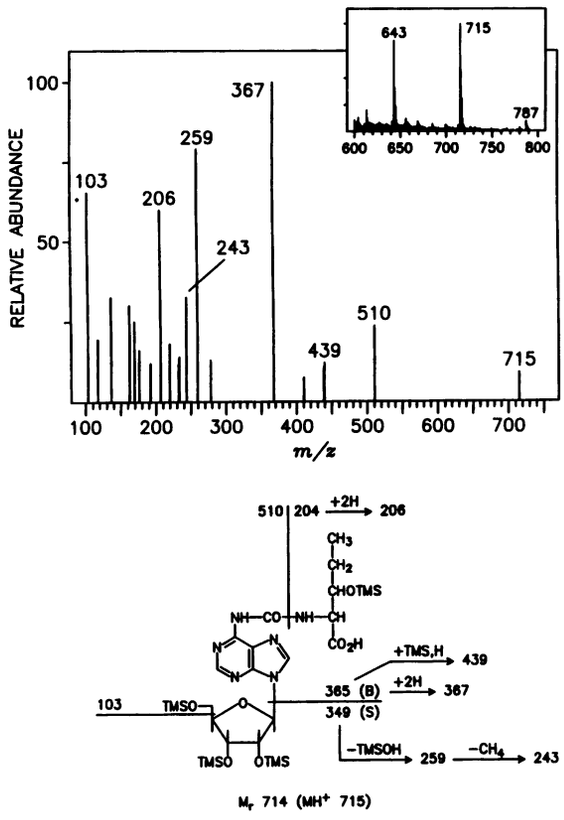


Figure 3. Mass spectrum of the products from collision-induced dissociation of the *m/z* 715 ion (MH^+) from the FAB mass spectrum of trimethylsilylated N27.4, with assignments shown on the accompanying structure. Inset: FAB mass spectrum of trimethylsilylated N27.4.

Table 1. Occurrence of nucleosides N27.4 (**1**) and N31.3 (**2**) in Transfer RNA[a]

Organism	1	2
Archaea		
Methanococcus vannielii	+	b
Methanolobus tindarius	+	+
Pyrobaculum islandicum	+	+
Pyrococcus furiosus	+	+
Pyrodictium occultum	+	
Thermococcus sp.	+	+
Thermodiscus maritimus	+	
Thermoproteus neutrophilus	+	
Bacteria		
Thermodesulfobacterium commune	+	+
Thermotoga maritima	+	b

[a]Additional organisms studied from which neither **1** nor **2** were detected include: *Acidianus infernus*, *Aquifex pyrophilus*, *Archaeoglobus fulgidus*, *Haloferax volcanii*, *Methanobacterium thermoautotrophicum*, *Sulfolobus solfataricus*, *Thermoplasma acidophilum*.
[b]Trace.

and N31.3 are observed in Figure 2. The ion abundance pattern shown in Figure 2B is very similar to that of t^6A (21), with the exception that the side chain ion *m/z* 120 (protonated threonine) from t^6A appears at *m/z* 134 in Figure 2B.

The number of exchangeable hydrogen atoms in ions represented in Figure 2 was determined from separate LC/MS experiments in which deuterated HPLC mobile phase was used (22). The resulting ion abundance patterns indicate shifts of +5 for *m/z* 134 in Figures 2A and 2B, representing incorporation of five deuterium atoms. The neutral molecule of M_r 133 represented in its protonated form as *m/z* 134 therefore contains four exchangeable hydrogens. The adenine and adenosine ion shifts (+4, +5 and +6, +7) are highly reproducible and are characteristic of adenosine derivatives (29), and are due to partial exchange of H-8 (which is slightly acidic) plus complete exchange at N and O and of the hydrogen of ionization. Analogous exchange behavior for *m/z* 134 is exhibited by nucleoside N31.3, implying a side chain of similar structure as in N27.4, but insufficient ion signals were obtained for the shifted *m/z* 182 and 314 ions in Figure 2B to permit shift patterns to be clearly assigned. Mass spectra of deuterium exchanged t^6A acquired during the same experiment represented in Figure 2A (retention time 10.0 min; data not shown) exhibited virtually identical side chain and base deuterium shift patterns as in the mass spectrum of N27.4.

Molecular weight determination and collision-induced dissociation mass spectrum of unknown nucleoside N27.4

The trimethylsilyl derivative of N27.4 isolated from *T. commune* tRNA was examined by FAB-MS to determine the relative molecular mass of the intact nucleoside. Three ions were observed (Figure 3 inset) whose values are consistent with protonated molecular ions (MH^+) of a nucleoside of M_r 426 bearing three

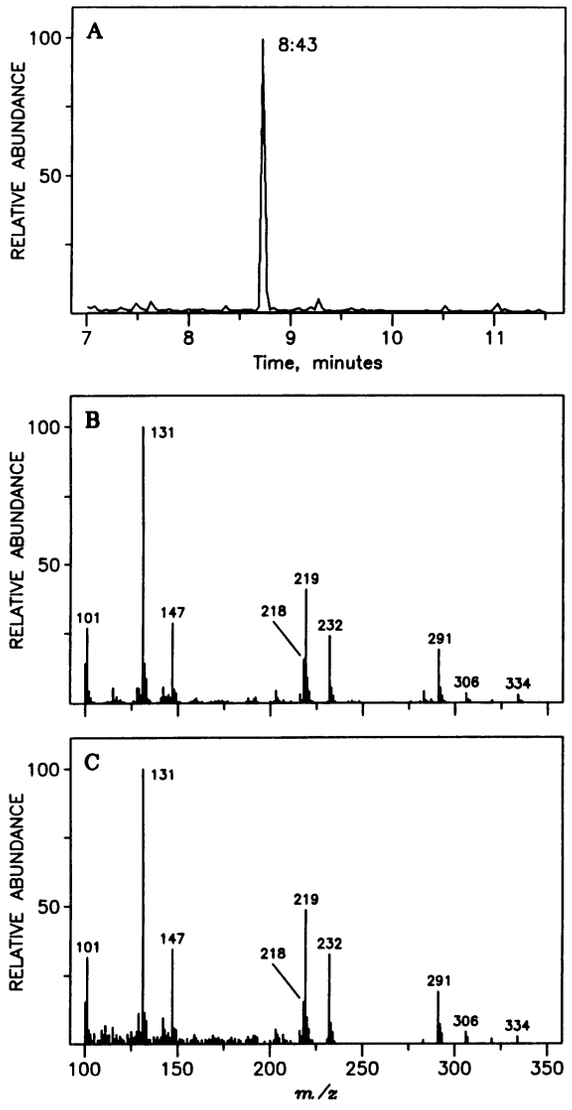


Figure 4. GC/EI-MS analysis of the alkaline hydrolysis products of nucleosides N27.4 and N31.3, following trimethylsilylation. (**A**) Reconstructed gas chromatogram based on the *m/z* 131 fragment ion from the amino acid of N27.4, showing an elution time of 8:43 ± 0.01 minutes (from expanded peak centroid, not shown). (**B**) Electron ionization mass spectrum of the amino acid derivative from N27.4, eluting at 8:43 minutes. Ion structure assignments are given in Table 2. (**C**) Electron ionization mass spectrum of the amino acid derivative from N31.3, eluting at 8:45 minutes (chromatogram not shown).

(*m/z* 643), four (*m/z* 715), and five (*m/z* 787) trimethylsilyl groups (net addition of 72 Da per silyl group). The MH^+ ion of the abundant *m/z* 715 species was submitted to collision-induced dissociation (30) to examine its constituent subunits. The resulting spectrum (Figure 3) reveals the most abundant ion in the spectrum to be *m/z* 367, assigned as the protonated base (BH), resulting from loss of a 348 Da ribosyl moiety from MH^+. Other prominent ions (*m/z* 103, 243 and 259) represent silylated ribose fragments (31). The prominent *m/z* 206 ion is assigned to the protonated amino acid side chain bearing one trimethylsilyl group and corresponds to the *m/z* 134 ion observed in the thermospray mass spectrum (Figure 2). The identity of the *m/z* 510 ion is assigned to loss of the neutral silylated amino acid (205 Da) from MH^+.

Table 2. Structure assignments for ions from mass spectra of the amino acid moiety of nucleosides N27.4 and N31.3 (Figures 4B and 4C), and threonine.

TMSO NHTMS
R-CH_2CH-CHCO_2TMS
4 3 2 1

	m/z	
	threonine[b]	N27.4 and N31.3 amino acid
Assignment[a]	R = H	R = CH_3
$R\text{-}CH_2CH{=}\overset{+}{O}TMS$	117	131
$TMS\text{-}NH{=}CHCO_2TMS^+$	218	218
TMSO R-CH_2CH-CH=$\overset{+}{N}$H-TMS	218	232
OH TMS-NHCH=$\overset{+\cdot}{C}$OTMS	219	219
TMSO TMS-NHCH=$\overset{+\cdot}{C}$OTMS	291	291
$M^+ - CH_3 - CO$	292	306
$M^+ - CH_3$	320	334

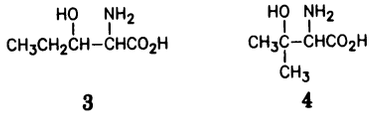
[a]TMS, trimethylsilyl; M, molecular ion.
[b]Assignments from ref. (32). Mass and abundance values from threonine-$(TMS)_3$ acquired in the present study are listed in the Results section.

Structure analysis by GC/EI-MS of amino acids released by alkaline hydrolysis of nucleosides N27.4 and N31.3

Separately isolated samples of nucleosides N27.1 and N31.3 were hydrolyzed by sodium hydroxide, and the products dried and then converted to volatile trimethylsilyl (TMS) derivatives to permit analysis by GC/EI-MS. The side chain amino acid of N27.4, bearing three TMS groups as determined by its mass spectrum, exhibited a gas chromatographic retention time of 8:43 minutes (Figure 4A), and the electron ionization mass spectrum shown in Figure 4B. An experimentally indistinguishable mass spectrum was produced by the side chain moiety from N31.3, Figure 4C.

The overall ion abundance pattern observed in Figures 4B and 4C are similar to the earlier reported spectrum of threonine-$(TMS)_3$ (32) and to the spectrum of silylated threonine acquired in this laboratory: *m/z* (relative abundance): 101(24), 117(92), 147(44), 218(100), 219(95), 291(39), 292(22), 320(7.9). The principal differences are 14 mass unit shifts of some ions as shown in Table 2. Structure assignments of ions from threonine are derived from the work of VandenHeuvel *et al.* (32) in which [2,3,4,4,4-$^2H_5$]threonine was used as a model. As is characteristic of the mass spectra of some TMS derivatives, no molecular ion is observed for threonine, so the molecular weight

values of 335 for threonine-TMS_3 and 349 for the tris-TMS derivative of the unknown amino acid are derived from the characteristic $M^+ - CH_3$ fragment ions resulting from loss of CH_3 from the TMS groups (33). The remaining ions demonstrate that the 14 Da difference between the two amino acids resides on C-3 or C-4, and not on C-1, C-2 or the amino nitrogen. The *m/z* 117 product of cleavage at C-2, C-3 of threonine shifts to *m/z* 131, while the products of H rearrangement (*m/z* 219) and TMS rearrangement (*m/z* 291) (32) are unchanged, further reflecting unaltered α-amino acid moieties. The *m/z* 218 ions from threonine represent two structurally different ions (Table 2), which assume different mass values in the case of the N27.4 and N31.3 amino acids, in



3 **4**

agreement with methylation at C-3 or C-4. The amino acid derived from nucleosides N27.4 and N31.3 is therefore assigned structure **3**. The stereochemistry at C-2 and C-3 was not addressed in the present study. Although the isomeric structure **4** could not be ruled out from the fragment ion assignments in Table 2, it was considered improbable because the tertiary carbon atom in **4** would likely lead to ion abundance patterns that would be markedly different from threonine.

The identity of **3** was tested by direct comparison of its mass spectrum and gas chromatographic retention time with those from authentic silylated 3-hydroxynorvaline, acquired in a subsequent GC/EI-MS experiment, under identical instrumental conditions as used for Figure 4. The result, shown in Figure 5, is a mass spectrum that is indistinguishable from those shown in Figures 4B and 4C. The retention time is identical to that shown in Figure 4A, and differs by 2 seconds from that of the amino acid derived from N31.3.

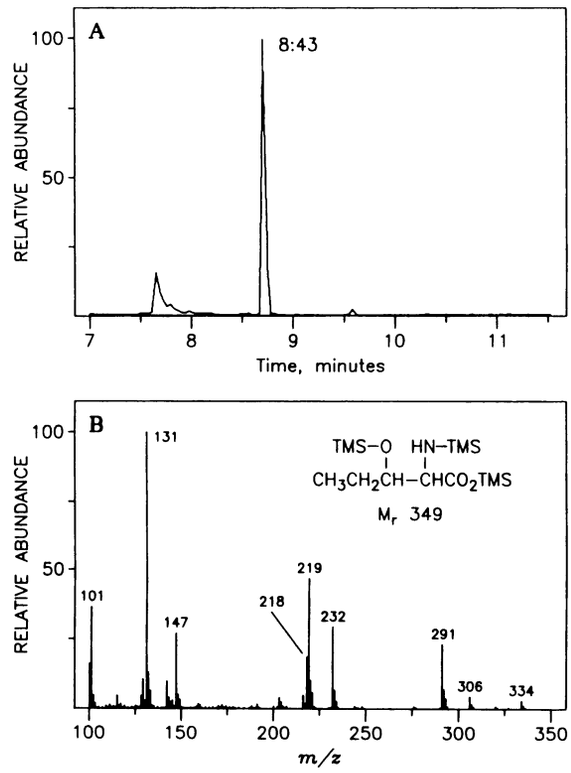


Figure 5. GC/EI-MS analysis of the *N,O*-tris(trimethylsilyl) derivative of authentic 3-hydroxynorvaline, for comparison with Figure 4. (**A**) Reconstructed gas chromatogram from *m/z* 131, showing elution time 8:43 ± 0.01 minutes (from expanded peak centroid, not shown). (**B**) Electron ionization mass spectrum.

Chemical synthesis of D,L-3-hydroxy-*N*-[[(9-β-D-ribofuranosyl-9*H*-purin-6-yl)amino]carbonyl]norvaline and comparison with nucleoside N27.4 using LC/MS

Synthesis of **1** was carried out by a coupling reaction between 3-hydroxynorvaline and the N^6-carbamoyl derivative of adenosine as outlined in Scheme 1, using a procedure adapted from ref (26). Synthetic **1** was compared against nucleoside N27.4 from *T. commune* by LC/MS, based on three criteria: HPLC retention times, which are generally reproducible to within ±0.25 minutes (21), UV absorbance ratios recorded from chromatographic peak profiles using a dual wavelength HPLC detector, and thermospray mass spectra. The retention time of synthetic **1** (arrow, Figure 1) is experimentally indistinguishable from that of N27.4, as is the thermospray mass spectrum (not shown). UV absorbance ratios (254 nm:280 nm, pH 6.0) were measured as 0.91 for synthetic **1**, and 0.84 ± 10% for N27.4 (measured from analysis shown in Figure 1).

Structural characterization of nucleosides N27.4 and N31.3

Compounds **1** and **2**, initially designated N27.4 and N31.3, were first detected during screening of an extensive collection of archaeal tRNAs (15) using thermospray LC/MS (21,28), a method which is significantly more definitive for identification of structurally known nucleosides in nucleic acid hydrolysates using HPLC than with UV detection alone. This technique (21) is particularly advantageous in providing limited structural information in the case of structural unknowns, and in generally not requiring chromatographic resolution of nucleosides in the enzymatic digests. As shown in Figure 2, nucleosides N27.4 and N31.3 produce mass spectra which exhibit ions characteristic of the adenosine moiety (adenine *m/z* 136, adenosine *m/z* 268) in Figure 2A, and of methylthioadenosine (*m/z* 182, 314) in Figure 2B. In addition, both mass spectra contain an *m/z* 134 fragment ion not previously reported in any nucleic acid constituent. The approximate retention times of 27 and 31 minutes do not correspond to known derivatives of adenosine or 2-methylthioadenosine, respectively (21), which led to the tentative conclusion that components N27.4 and N31.3 are previously unreported derivatives of adenosine. The abundance ratio of *m/z* 134:*m/z* 136 in Figure 2A is similar to that of *m/z* 120:*m/z* 136 from t^6A, in which *m/z* 120 is due to protonated threonine (21), and is thus consistent with the assignment of *m/z* 134 to a methylthreonine isomer. It is noted that the t^6A analog m^6t^6A (*N*-[[(9-β-D-ribofuranosyl-9*H*-purin-6-yl)methylamino] carbonyl]threonine) (34), although an isomer of nucleoside N27.4, is excluded due to HPLC retention time (27.0 min (21)) and the absence of methyladenine ions (*m/z* 150, 282 expected) in Figure 2A. The absence of a molecular ion in Figure 2 which is characteristic of some hypermodified nucleosides, including t^6A (21), prevents further conclusions to be reached from these data alone.

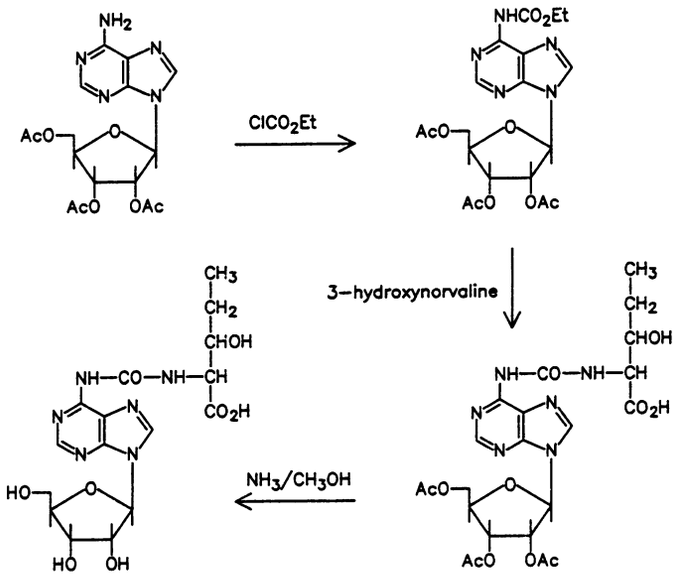
The number of hydrogen atoms exchangeable by deuterium in the ion species represented in Figure 2 was determined by an LC/MS method in which deuterated HPLC solvents are utilized (22). The results showed that the *m/z* 134 ion contains the same number of exchangeable hydrogen atoms as threonine, four (five including the deuteron of ionization), and is thus consistent with a C-methyl analog of threonine and rigorously excludes O- or N-methylation which would have resulted in a mass shift of +4 in Figure 2.

No ions representing intact N27.4 and N31.3 were visible in the thermospray mass spectra (Figure 2), so an alternative method (FAB-MS) was used to determine the relative molecular masses of the intact molecules. Trimethylsilylation has been shown to enhance sensitivity of FAB analyses (35), so N27.4 (about 4-fold more abundant than N31.3) was examined as its TMS derivative. Reaction conditions were milder than typically used (36) due to the tendency of ureidopurine nucleosides to dehydrate during silylation (37). If the *m/z* 134 and *m/z* 268 species were bound directly together by a covalent bond, the M_r of the intact molecule is predicted to be 398 Da (267 + 133−2H), which would give 614, 686 and 758 Da for the tris-, tetra- and penta-trimethylsilyl derivatives, respectively. Three ions (*m/z* 643, *m/z* 715 and *m/z* 787) are seen in the upper mass range of the FAB spectrum (inset, Figure 3) which differ by 72 u, characteristic of differences in the extent of silylation, indicating derivatives of M_r 642, 714 and 786, respectively. Subtraction of multiple 72 Da units thus leads to a M_r of 426 for nucleoside N27.4. This value is 28 Da greater than expected for simple adduction of an adenosine moiety by an amino acid of M_r 133, and suggests the possibility that the amino acid is adducted through a carbamoyl linkage to the purine ring, and that N27.4 and N31.3 are structural analogues of t^6A.

Additional evidence was provided from collision-induced dissociation of the protonated tetra-trimethylsilylated species, *m/z* 715 by FAB-MS/MS. Most ions in the product ion spectrum in Figure 3, assigned in the Results section, are readily identifiable from known fragmentation reactions of silylated nucleosides (31). Two abundant fragment ions, *m/z* 206 and *m/z* 510 provide conclusive evidence for location of the additional 28 Da species. The latter ion is consistent with loss of the intact mono-silylated amino acid (205 Da) from MH^+. In addition, no ions representing loss of 28 u from either MH^+, BH or the protonated amino acid ion (*m/z* 206) are observed. Taken together, these observations suggest that the 28 Da subunit is associated with the adenine moiety or forms a bridge between the adenosine and amino acid moieties. The latter molecular arrangement, when the 28 Da unit corresponds to a carbonyl function, has ample precedent in tRNA in the carbonyl-linked threonyl derivatives of adenosine, t^6A (2′), of N^6-methyl-adenosine, m^6t^6A (34) and of 2-methylthioadenosine, ms^2t^6A (38).

Given the evidence that N27.4 could be an analogue of t^6A, it was judged that the amino acid would be amenable to independent structural characterization following hydrolysis from the nucleoside. The method employed was GC/EI-MS of the volatile trimethylsilyl derivative of the amino acid released by alkaline hydrolysis (39), which can be carried out at the sub-microgram level and provides an extensive and informative set of fragment ions. Assignments for ions in the resulting mass spectrum (Figure 4B, 4C and Table 2) are consistent with the amino acid structure **3**, although it was not possible to unambiguously exclude **4**, which also fits the structural criteria imposed from assignments in Table 2 and from deuterium exchange results in Figure 2. The amino acid structure was conclusively established as **3** by comparison of gas chromatographic retention times and electron ionization mass spectra of the amino acids from N27.4 and N31.3 with authentic 3-hydroxynorvaline (Figure 4 vs. 5). The GC/EI-MS method is viewed as the single most definitive test of structural identity, because of the great precision of retention time measurement by gas chromatography (within ±2 seconds) and the great reproducibility of EI mass spectra, which are highly sensitive to structural variations in linear molecules such as amino acids.

Although the carbamoyl-linked nucleoside structures **1** and **2** are strongly inferred by the foregoing data and the biological precedent of the t^6A family of nucleosides (24,34,38) final structure proof was obtained by chemical synthesis of **1**, by the procedure summarized in Scheme 1, and direct comparison with



Scheme 1.

the nucleoside N27.4 in an enzymatic hydrolysate of *T. commune* tRNA (Figure 1, arrow). Nucleoside N31.3 is concluded to have structure **2**, based principally on the structural identity of its amino acid side chain and that of authentic 3-hydroxynorvaline, and appropriate mass shifts in comparison of mass spectra in Figure 2A and B. In addition, the UV 254 nm:280 nm absorbance ratio of N31.3 (1.1 ± 25%) is qualitatively similar to that of ms^2t^6A (1.3), within the limitations of peak height data for **2** in Figure 1 and organic mobile phase differences between elution points of ms^2t^6A and **2**.

DISCUSSION

The two nucleosides characterized in the present study, hn^6A and ms^2hn^6A, represent new N^6-carbonyl derivatives of adenosine, and their identities raise issues of occurence, biosynthesis and function. Other naturally-occurring members of this nucleoside family are t^6A (40), m^6t^6A (34) and ms^2t^6A (38). The latter three nucleosides occur in tRNA from all three primary phylogenetic domains, and when observed in sequenced tRNAs (41), they always occur in position 37, immediately adjacent to the 3 end of the anticodon, and generally, but not exclusively, in tRNAs which translate ANN codons. Although

not yet reported in any sequenced tRNA, it is reasonable to presume that hn^6A and ms^2hn^6A will also occur in position 37. Unlike t^6A, m^6t^6A and ms^2t^6A, however, presence of hn^6A and ms^2hn^6A is presently limited to tRNA from thermophilic bacteria and certain archaea (principally thermophiles), as shown in Table 1.

Although no information is available regarding biosynthesis of hn^6A and ms^2hn^6A, consideration of the biosynthetic pathway for t^6A is informative. The latter nucleoside is synthesized by sequential addition of the elements of CO and threonine to adenosine (42à45) at the tRNA level (44,45), in an ATP-dependent reaction (44,45). A single protein apparently mediates addition of both CO (from bicarbonate) and threonine (45). The same enzyme preparation also accepts glycine as a substrate and synthesizes the corresponding glycine analogue, g^6A (45), which was characterized in unfractionated tRNA from yeast, in trace amount (46). It is reasonable to assume that hn^6A (and probably ms^2hn^6A), by analogy with t^6A, are synthesized by post-transcriptional addition of CO and an amino acid to adenosine in tRNA. Origin of the 3-hydroxynorvaline substituent is, however, of greater interest, because it is not a common α-amino acid. Whether it is added directly to the N^6-aminocarbonyl functional group in adenosine, or converted *in situ* from g^6A, t^6A or another (hypothetical) precursor remains to be established.

Final issues concern the occurrence and function of hn^6A and ms^2hn^6A in tRNA. If they may be considered analogues of t^6A in which the 3-hydroxynorvalyl substituent adopts a conformation similar to that found for threonine in the crystal structure of t^6A (47), and if they likewise occur adjacent to the tRNA anticodon (position 37), then they may serve some of the same proposed roles. Various studies of t^6A have led to the suggestion that the nature of the modification serves to enhance fidelity of mRNA translation by preventing wobble on the 3-side of the anticodon as a consequence of reduced flexibility through more highly stacked (stabilized) structure (of Upt^6A relative to UpA) (48), or from shielding of the base-pairing sites of the adenine moiety by conformational folding of the amino acid substituent (47). Fully-modified *E.coli* $tRNA^{Ile}$ binds more efficiently to ribosomes than the unmodified tRNA, in the presence of Mg^{++}, and it was suggested that the t^6A substituent strengthens this interaction by facilitating a Mg^{++} bridge between tRNA and the ribosome (49). Any or all of these functions could be served by hn^6A or ms^2hn^6A.

It has been proposed that early life developed in a thermophilic environment (50,51), and it is clear from recent evidence that most hyperthermophiles are found in the archaeal domain (51). Of interest, therefore, is whether 3-hydroxynorvaline in these nucleosides enhances the stability of tRNA and provides an advantage for growth at elevated temperature, relative to t^6A-containing tRNAs. This view is consistent with occurrence of hn^6A and ms^2hn^6A primarily in the hyperthermophilic organisms examined; their presence in the mesophiles *M. vannielii* and *M. tindarius* growing at temperatures significantly lower than the two thermophilic eubacteria (*T. commune*, optimum 70°C, and *T. maritima*, 80°C) could merely reflect their archaeal lineage. Both *T. maritima* and *T. commune* are phylogenetically among the most deeply branched bacteria (50,52) and as such are more closely related to the archaeal thermophiles than most other bacteria (51). Although *M. vannielii* grows optimally at 37° it is noted that other members of the order *Methanococcales* are hyperthermophiles (53). On the other hand, hn^6A and ms^2hn^6A do not replace t^6A or ms^2t^6A, but in fact, are typically present in lower amounts relative to them (assuming equivalent molar absorptivities; see Figure 1). So it is also possible that hn^6A and ms^2hn^6A may serve no direct role in tRNA thermal stabilization, but as has for example been shown for presence of i^6A-37 family of nucleosides in *S. typhimurum* tRNA, may serve potential regulatory roles in gene expression (54,55).

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