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Structures of condensed tricyclic nucleosides of phenylalanine transfer ribonucleic acids

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Abstract

The unstable minor nucleosides, wyosine, wybutosine, and β-hydroxywybutosine, were isolated from tRNAs in sufficient amounts for determination of their structures. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: amino acids; amino acid derivatives; nucleosides; configuration; Heck reaction.

The structures **2a,b** have been assigned to wyosine and wybutosine isolated from yeast $tRNAs^{Phe}$ and these compounds have already been synthesized.[1,2] However, lack of samples of these nucleosides from $tRNAs^{Phe}$ has hampered precise identification of the position of glycosylation and the structure of the sugar moiety. On the other hand, we envisioned **1c,d** as the most probable alternatives for the minor base β-hydroxywybutine from rat liver $tRNA^{Phe}$ and have already synthesized these two candidates[3] and their 3-β-D-ribofuranosides **2c,d**.[4] Unfortunately, solution of the structural problems regarding the minor base and its nucleoside β-hydroxywybutosine has also had to await isolation of samples from $tRNA^{Phe}$. This paper reports the isolation of these nucleosides from tRNAs in sufficient quantities for identification with synthetic samples. Thus, the structures **2a,b,d** have been unambiguously assigned to wyosine, wybutosine, and β-hydroxywybutosine, respectively.

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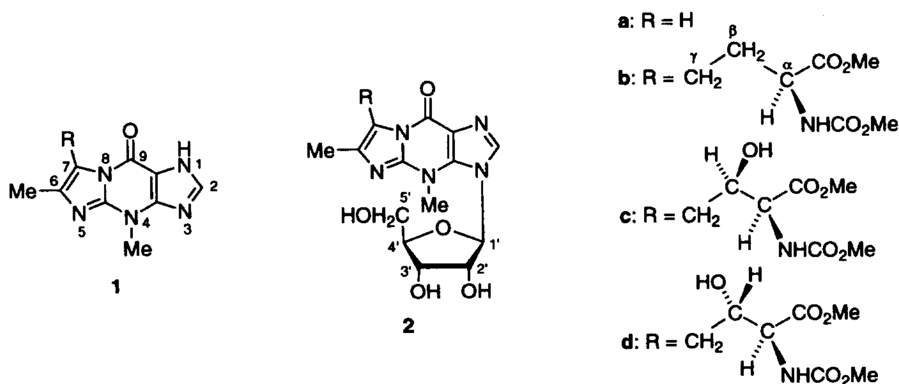


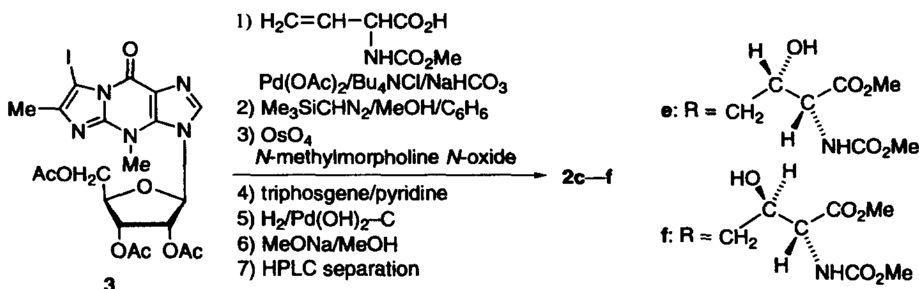
Wyosine: Takemura's group obtained a mixture containing wyosine 5′-mononucleotide by digestion of the wyosine-containing hexanucleotide, which was obtained from torula yeast $tRNA^{Phe}$ by treatment with RNase T_1/RNase A, with snake venom phosphodiesterase after removal of the 3′-terminal phosphate with alkaline phosphatase; the mixture was dephosphorylated with *Escherichia coli* alkaline phosphatase, and then chromatographed two-dimensionally on a cellulose plate.[5] Unfortunately, the amount (0.13 A_{260} unit;[5] estimated to be ca. 8 μg) of wyosine thus obtained was inadequate for gathering information other than the UV spectrum and the chromatographic behavior at that time. On the other hand, McCloskey's group isolated a minor nucleoside from unfractionated archaebacterial tRNA by digestion using nuclease P_1 and alkaline phosphatase followed by reversed-phase HPLC, and they proposed its structure to be **2** (R=Me).[6] This procedure appeared more convenient to us for the purpose of large-scale isolation of wyosine. Because the glycosyl bond of wyosine or its congeners is highly sensitive to hydrolysis[1,2] and because nucleosides are hardly soluble in an organic solvent such as $CDCl_3$, we planned to identify these nucleosides through their more stable[7] 2′,3′,5′-*O*-triacetates. We preferred triacetyl-**2a**-d_9 to triacetyl-**2a** in order to rule out the possibility that wyosine might be monoacetyl-**2a**. Authentic triacetyl-**2a**-d_9 {CD (H_2O) $\Delta\varepsilon_{232}$ +3.51; ^{1}H NMR ($CDCl_3$) δ: 2.33 (d, J=1 Hz, CMe), 4.31 and 4.33 [dd, J=12.7 and 2.4 Hz, C(5′)-H_2], 4.51 [dt, J=3.9 and 2.4 Hz, C(4′)-H], 5.50 [dd, J=5.5 and 3.9 Hz, C(3′)-H_2], 5.85 [dd, J=6 and 5.5 Hz, C(2′)-H], 6.23 [d, J=6 Hz, C(1′)-H], 7.44 [br, C(7)-H], 7.77 [s, C(2)-H]} was prepared in 93% yield by treatment of **2a**[1a] with Ac_2O-d_6 in pyridine at room temperature for 4 h. On the other hand, unfractionated tRNA (1 g, 15400 A_{260} units) obtained from dry torula yeast (*Candida utilis* purchased from Sigma Chemical Co.) was treated with nuclease P_1 (490 units) in 20 mM acetate buffer (pH 5.3) at 50°C for 3 h. It was found that the amount of the enzyme was insufficient for liberation of wyosine monophosphate. The hydrolysate was then subjected to column chromatography [Cosmosil® 140C_{18}-OPN; H_2O and then MeOH:H_2O (30:70, v/v)]. The MeOH–H_2O fraction (390 A_{260} units) was further digested with nuclease P_1 (1500 units) (at 50°C for 11 h) and then with calf intestinal alkaline phosphatase (10 units) at pH 9 and 50°C for 2 h. The nucleoside mixture thus obtained was subjected to HPLC [Hibar LiChrosorb® RP-18; MeOH:H_2O (30:70, v/v)], providing wyosine (1 A_{310} unit, ca. 70 μg), the HPLC behavior of which was identical to that of **2a**. The nucleoside was converted into the triacetate-d_9, which was identical to triacetyl-**2a**-d_9 on the basis of MS, 500 MHz ^{1}H NMR, and CD spectra. The structure of wyosine was hereby determined to be **2a**.

Wybutosine: This compound has been isolated by enzymatic digestion (pancreatic RNase/snake venom phosphodiesterase/RNase T_2[8] or pancreatic RNase/phosphomonoesterase/RNase T_1/snake venom phosphodiesterase/phosphomonoesterase[9]) of baker's[8] or brewer's[9] yeast $tRNA^{Phe}$ followed by chromatographic separation. Although Blobstein et al. isolated wybutosine on a scale of 90 μg according to the former procedure,[10] full characterization of this compound was difficult at that time. We obtained

this compound (ca. 50 μg), the HPLC behavior of which was identical with that of **2b**,[2a] from dry baker's yeast (*Saccharomyces cerevisiae* Type I purchased from Sigma Chemical Co.) (150 g) in a manner similar to that described above for the isolation of wyosine. Identity of the triacetate-d_9 of wybutosine with authentic triacetyl-**2b**-d_9 {^{1}H NMR ($CDCl_3$) δ: 2.05 and 2.16 [m, C(β)-H_2], 2.22 (s, CMe), 2.99 (ddd, *J*=14.6, 5.4 and 10 Hz) and 3.31 (ddd, *J*=14.6, 4.9 and 10.5 Hz) [C(γ)-H_2], 3.70 and 3.71 (s, two OMes), 4.12 (s, NMe), 4.32 [m, C(α)-H and C(5')-H_2], 4.50 [dt, *J*=3.9 and 2.5 Hz, C(4')-H], 5.49 [dd, *J*=5.5 and 3.9 Hz, C(3')-H], 5.83 [dd, *J*=6 and 5.5 Hz, C(2')-H], 5.86 (d, *J*=8 Hz, NH), 6.19 [d, *J*=6 Hz, C(1')-H], 7.73 [s, C(2)-H]} was established by comparison of the MS and ^{1}H NMR spectra and chromatographic behavior. As *S* configuration has already been assigned to the base wybutine (**1b**),[11] an alternative structure to be eliminated was β-L-ribofuranosyl-**1b**. Although this diastereomer itself is not available, the HPLC [Hibar LiChrosorb® RP-18; MeOH:H_2O (40:60, v/v)] behavior of its enantiomer β-D-ribofurranosyl-*ent*-**1b** obtained from the triacetate[2a] was different from that of wybutosine, confirming that **2b** is the correct expression for wybutosine.

β-Hydroxywybutosine: Crude tRNA (100 mg, 1000 A_{260} units) obtained from rat liver (176 g) was incubated at pH 2.9 and 40°C for 16 h. Precipitation by addition of EtOH and centrifugation followed by preparative TLC [silica gel; $CHCl_3$:MeOH (10:1, v/v)] afforded a few micrograms of β-hydroxywybutine, which could be discriminated from **1c**[3] (by means of HPLC[3] and ^{1}H NMR spectroscopy) and was identical with **1d**.[3] The structure **1d** was also found to represent the absolute configuration of the minor base on the basis of chiral HPLC[12] analysis. Kasai et al. reported the coexistence of a minor fluorescent substance, the R_f value of which corresponded to **1b**, with β-hydroxywybutine in the hydrolysate of rat liver $tRNA^{Phe}$.[13] In the present experiment, however, no trace of **1b** was found in the hydrolysate. In a separate run, crude tRNA obtained from rat liver (787 g) was purified by a DEAE–cellulose column [eluted with 0.02 M Tris buffer (pH 7.5)–0.01 M $MgCl_2$ and then with 1 M NaCl–0.02 M Tris buffer (pH 7.5)–0.01 M $MgCl_2$]. Unfractionated tRNA (350 mg, 5250 A_{260} units) thus obtained was treated with nuclease P_1 (500 units), and the hydrolysate was purified on Cosmosil® in a manner similar to that described above for the isolation of wyosine. The product (210 A_{260} units) was treated again with nuclease P_1 (2000 units) at 50°C for 3 h. The resulting mononucleotides were digested with alkaline phosphatase, and the mixture was subjected to HPLC as described above to afford β-hydroxywybutosine (1.5 A_{310} units, ca. 100 μg) for the first time. The HPLC behavior of this nucleoside was identical with that of **2d**. The ^{1}H NMR spectrum of the tetra-*O*-acetyl-d_{12} compound prepared from β-hydroxywybutosine was identical with that of tetraacetyl-**2d**-d_{12} {^{1}H NMR ($CDCl_3$) δ: 2.24 (s, CMe), 3.01 (dd, *J*=15.1 and 8.8 Hz) and 3.89 (dd, *J*=15.1 and 4.4 Hz) [C(γ)-H_2], 3.69 and 3.70 (s, two OMes), 4.11 (s, NMe), 4.32 [m, C(5')-H_2], 4.50 (m, C(4')-H), 4.71 [dd, *J*=8.8 and 5.4 Hz, C(α)-H], 5.48 [dd, *J*=5.4 and 3.4 Hz, C(3')-H], 5.50 [m, C(β)-H], 5.83 [dd, *J*=6.4 and 5.4 Hz, C(2')-H], 6.12 (d, *J*=8.8 Hz, NH), 6.19 [d, *J*=6.4 Hz, C(1')-H], 7.74 [s, C(2)-H]}. Evidence to rule out 3-β-L-ribofuranosyl-**1d** for the structure of β-hydroxywybutosine was obtained by the synthesis of **2f**, the enantiomer of 3-β-L-ribofuranosyl-**1d**, as delineated below. According to the procedure for the synthesis of **2b**,[2a] the iodide **3** was subjected to the Heck reaction with (±)-*N*-(methoxycarbonyl)vinylglycine[14] followed by methylation to produce a diastereomeric mixture of the β,γ-unsaturated amino acid derivatives. Dihydroxylation of the mixture followed by cyclocondensation and hydrogenolysis in the manner established for the synthesis of **2c,d**[4] afforded a mixture of the protected nucleosides. Deacetylation and HPLC separation [Hibar LiChrosorb® RP-18; MeOH:H_2O (30:70, v/v)] of the mixture provided the four diastereomers **2c–f**. The HPLC behavior of β-hydroxywybutosine was different from that of **2f** {^{1}H NMR [$(CD_3)_2CO$] δ: 2.24 (s, CMe), 3.18 [dd, *J*=14.7 and 8.3 Hz, one of C(γ)-H_2], 3.64 and 3.67 [s, overlapping with a multiplet arising from one of C(γ)-H_2, two OMes], 3.84 and 3.92 [m, C(5')-H_2], 4.21 [m, C(4')-H], 4.23 (s, NMe), 4.28 [m, C(β)-H], 4.37 [m, C(α)-H], 4.49 [m, C(3')-H and C(5')-

OH], 4.58 [br, C(β)-OH], 4.74 [m, C(2′)-H], 4.90 and 5.25 [br, C(3′)-OH and C(2′)-OH], 6.30 [d, J=4.9 Hz, C(1′)-H], 6.68 (d, J=7.8 Hz, NH), 8.21 [s, C(2)-H]}, allowing us to conclude that the structure of β-hydroxywybutosine is **2d**.



In conclusion, our synthetic samples of **1** and **2** greatly helped toward isolation of the nucleosides under consideration and hence determination of their complete structures.

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