Structure of Wyosine, the Condensed Tricyclic Nucleoside of Torula Yeast Phenylalanine Transfer Ribonucleic Acid

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Received November 21, 2001; accepted January 11, 2002

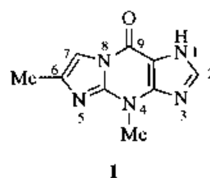
Large-scale isolation of the minor nucleoside wyosine of torula yeast tRNA^{Phe} was accomplished by a combination of enzymatic digestion and reversed-phase chromatography: the wyosine-containing nucleotide fraction, which was obtained by partial digestion of unfractionated tRNA (1 g) with nuclease P_1, was concentrated by reversed-phase column chromatography followed by complete digestion with nuclease P_1/alkaline phosphatase. The nucleoside mixture thus obtained was purified by reversed-phase HPLC, providing wyosine (70 μg). Comparison of this nucleoside with a chemically synthesized authentic sample has unambiguously established that the structure of wyosine is 4,6-dimethyl-3-β-D-ribofuranosyl-3,4-dihydro-9*H*-imidazo[1,2-*a*]purin-9-one (2).

Key words wyosine; minor nucleoside; torula yeast tRNA^{Phe}; fluorescent nucleoside; condensed tricyclic nucleoside; ^{1}H-NMR

A fluorescent component adjacent to the 3′-end of the anticodon of torula yeast tRNA^{Phe} was isolated as the nucleoside wyosine,[1)] and the structure of its base wye has been determined to be **1**.[1b,2)] It has been proposed by comparison of the chemical properties and UV spectra of wyosine with those of model compounds that 3-β-D-ribofuranosylwye (**2**) is the most probable structure for this nucleoside.[1b)] However, Reese and Whittall have claimed that wyosine is unlikely to be a ribonucleoside on the basis of the rate studies on the hydrolysis of model compounds.[3)] As wyosine was isolated from the tRNA in only a minute quantity (0.13 A_{260} unit[1b)]; estimated to be *ca.* 8 μg), precise identification of the position of glycosylation and the structure of the sugar moiety has to rest on chemical synthesis. We and other groups have already synthesized the target molecule **2**.[4)] However, lack of a sample of wyosine has hampered its structural determination. As the exceptional susceptibility to hydrolysis of wyosine[1a)] had been anticipated to aggravate the difficulties of its isolation, we carried out rate studies[4c,5)] on the hydrolysis of **2**, disclosing that **2** can be handled at pH 5—10 at 37 °C without suffering a heavy loss in quantity. Being encouraged by this knowledge, we started the present investigation. A preliminary communication of a part of this work has been published.[6)]

In order to avoid difficulties presented by the instability and the poor solubility of **2** in an organic solvent such as $CDCl_3$, we planned to identify the nucleoside through its more stable[7)] 2′,3′,5′-*O*-triacetate. We preferred triacetyl-d_9-**2** (**3**) to triacetyl-**2** in order to rule out a slim possibility that wyosine is an acetylated **2** by nature. An authentic sample of **3** was prepared in 93% yield by treatment of **2**[4c)] with Ac_2O-d_6 in pyridine at room temperature for 4 h. At the outset of this work we wished to determine a minimum amount of **3** required for obtaining a satisfactory ^{1}H-NMR spectrum by routine measurement with a 500 MHz instrument and found that 50 μg was enough to locate every proton of **3**. Pretreatment of the solvent $CDCl_3$ with aluminum oxide was of prime importance for getting a good spectrum, otherwise poor reproducibility of the chemical shifts was obtained probably owing to a trace of DCl contaminated.

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 alkaline phosphatase, and then chromatographed two dimensionally on a cellulose plate.[1)] Later on, McCloskey's group isolated a minor nucleoside from unfractionated archaebacterial tRNA's by digestion using nuclease P_1 and alkaline phosphatase followed by reversed-phase HPLC, and they proposed its structure to be 7-methyl-**2**.[8)] This procedure appeared more convenient to us. However, the use of the amount of the enzyme according to the reported procedure (4 units/100 μg tRNA)[9)] is not practical for large-scale isolation of wyosine. Thus unfractionated tRNA (1 g) obtained from dry torula yeast was treated at first with a much smaller amount of nuclease P_1 (490 units) at pH 5.3 and 50 °C for 3 h. Hydrolysis of the phosphodiester linkages was incomplete under these conditions, liberating little wyosine mononucleotide. The hydrolysate was then purified by reversed-phase column chromatography. The column was washed with water to remove a large amount of hydrophilic components. The lipophilic substances retained on the column were then eluted with methanol–water (30 : 70, v/v). The aqueous methanol fraction was further digested with nuclease P_1 (1500 units) (at pH 5.3 and 50 °C for 11 h) and then with alkaline phosphatase. The mixture of nucleosides thus obtained was purified by reversed-phase HPLC, providing wyosine (1 A_{310} unit, *ca.* 70 μg), of which HPLC behavior was identical with that of **2**. The nucleoside was converted into the triacetate-d_9, which was identical with **3** on the basis of MS, ^{1}H-NMR, and CD spectra. The structure of wyosine was hereby determined to be 4,6-dimethyl-3-β-D-ribofuranosyl-3,4-dihydro-9*H*-imidazo[1,2-*a*]purin-9-one (**2**).



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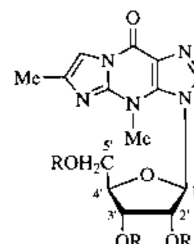

2: R = H
3: R = CD_3CO

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Experimental

General Notes Spectra reported herein were recorded on a JEOL JMS-SX102A mass spectrometer, a Hitachi model 320 UV spectrophotometer, a JEOL JNM-GSX-500 NMR spectrometer (measured at 25 °C with Me_4Si as an internal standard), and a JASCO J-700 spectropolarimeter. MS measurements were performed by Dr. M. Takani and her associates at Kanazawa University. The optical rotation was measured with a JASCO DIP-181 polarimeter using a 10 cm sample tube. The HPLC system employed consisted of a Tosoh CCPD pump, an injection valve unit, a UV-8020 detector (operated at 310 nm), and a Chromatocorder 21 integrator or a Waters 6000A pump, a U6K injector, and a model 440 absorbance detector (operated at 254 nm). The following abbreviations are used: br=broad, d=doublet, dd=doublet-of-doublets, ddd=doublet-of-doublets-of-doublets, q=quartet, s=singlet.

4,6-Dimethyl-3-[2,3,5-tri-*O*-(acetyl-d_3)-β-D-ribofuranosyl]-3,4-dihydro-9*H*-imidazo[1,2-*a*]purin-9-one (3) Ac_2O-d_6 (0.15 ml) was added to a suspension of **2**[4c] (50 mg, 0.15 mmol) in dry pyridine (0.5 ml), and the mixture was stirred at room temperature for 4 h. The resulting solution was concentrated *in vacuo*, and the residue was dissolved in CH_2Cl_2 (15 ml). The solution was washed successively with 10% aqueous citric acid (2×15 ml), H_2O (20 ml), saturated aqueous $NaHCO_3$ (2×15 ml), and H_2O (20 ml), dried over $MgSO_4$, and concentrated *in vacuo*, leaving **3** (65 mg, 93%) as a colorless oil, $[\alpha]_D^{16}$ −23° (c=9.76, MeOH). CD (c=2.69×10^{-5} M, H_2O) $\Delta\varepsilon$ (nm): +3.51 (232). MS *m/z*: 470 (M^+). UV nm (ε): $\lambda_{max}^{95\%\ EtOH}$ 236 (27400), 291 (7100); $\lambda_{max}^{H_2O}$ (pH 2) 230 (30600), 275 (10000); $\lambda_{max}^{H_2O}$ (pH 7) 237 (30300), 294 (6900); $\lambda_{max}^{H_2O}$ (pH 13) 235 (31800), 294 (7100). ^{1}H-NMR ($CDCl_3$) δ: 2.33 (3H, d, J=1 Hz, CMe), 4.18 (3H, s, NMe), 4.31, 4.33 [1H each, dd, J=12.7, 2.4 Hz, C(5′)-H_2], 4.51 [1H, ddd, J=3.9, 2.4, 2.4 Hz, C(4′)-H], 5.50 [1H, dd, J=5.5, 3.9 Hz, C(3′)-H], 5.85 [1H, dd, J=6, 5.5 Hz, C(2′)-H], 6.23 [1H, d, J=6 Hz, C(1′)-H], 7.44 [1H, q, J=1 Hz, C(7)-H], 7.77 [1H, s, C(2)-H].

Isolation and Identification of Wyosine Unfractionated tRNA was obtained as described below in the usual manner.[10] To a suspension of dry *Candida utilis* (Sigma Chemical) (890 g) in H_2O (27 l) was added 88% (v/v) aqueous phenol (13.5 l), and the mixture was shaken for 3 h. The mixture was centrifuged (8000 rpm, 25 min) after storage at 4 °C overnight. The aqueous layer (24 l) was mixed with NaCl (710 g) and $MgCl_2 \cdot 6H_2O$ (99 g), and then EtOH (24 l) was added to the resultant solution. The mixture was centrifuged (15 min), and the precipitate was suspended in H_2O (510 ml). The suspension was centrifuged, and cetyltrimethylammonium bromide (43 g) was added to the stirred supernatant fraction (310000 A_{260} units). The resulting precipitate was collected by centrifugation, dissolved in 1 M NaCl–0.01 M $MgCl_2$–H_2O (1 l), and precipitated again by addition of EtOH (1 l). The last operation was repeated two more times. The precipitate thus obtained was washed successively with 67% aqueous EtOH (containing 0.1 M NaCl–0.005 M $MgCl_2$), EtOH, and Et_2O, and dried over P_2O_5, giving crude tRNA (12.4 g, 125000 A_{260} units). This product (8.06 g) was dissolved in Tris buffer [0.02 M $(HOCH_2)_3CNH_2$–HCl (pH 7.5) containing 0.01 M $MgCl_2$] and subjected to a column packed with ion exchange cellulose (Whatman DE 32) (dry weight 75 g), which had been equilibrated with the Tris buffer. The column was washed with the Tris buffer (200 ml) followed by elution with the Tris buffer (2 l) containing 1 M NaCl at the rate of 300 ml per hour. EtOH (4 l) was added to the eluate, and the precipitate that separated was collected by centrifugation, washed successively with 67% aqueous EtOH (containing 0.1 M NaCl–0.005 M $MgCl_2$), EtOH, and Et_2O, and dried over P_2O_5, providing tRNA (4.00 g, 61600 A_{260} units).

This product (1 g) was treated with nuclease P_1 (Yamasa) (1 mg, 490 units) in 0.02 M acetate buffer (pH 5.3, 100 ml) at 50 °C for 3 h. The hydrolysate was then purified by column chromatography on Cosmosil 140C_{18}-OPN (Nacalai Tesque) (45 g) [H_2O (240 ml) and then MeOH–H_2O (30 : 70, v/v; 140 ml)]. The MeOH–H_2O fraction was concentrated *in vacuo* to leave a glass. It was found by HPLC analysis that treatment of a small portion of this product with alkaline phosphatase at pH 9 liberated no wyosine. The rest of the nucleotide mixture (390 A_{260} units) was dissolved in H_2O (7.7 ml), and a solution of nuclease P_1 (3.1 mg, 1500 units) in 0.02 M acetate buffer (pH 5.3, 9.6 ml) was added. The whole was kept at 50 °C. At intervals, aliquots (20 μl) of the solution were withdrawn, made alkaline by addition of 0.5% aqueous NH_3 (3.5 μl), and treated with alkaline phosphatase. Quantitative analysis of wyosine thus formed by HPLC showed that it took 6 h to complete the digestion. After 11 h, 2% aqueous NH_3 (0.76 ml) and *Escherichia coli* alkaline phosphatase (Takara Shuzo) (10 units) were added, and the mixture (pH 9) was kept at 50 °C for 2 h. The reaction mixture was neutralized with 1 N aqueous HCl and concentrated *in vacuo* to 5 ml. This was purified by HPLC [LiChrosorb RP-18 (7 μm, 250×10 mm) (Merck); MeOH–H_2O (30 : 70, v/v)] in four portions, providing wyosine (1 A_{310} unit, *ca.* 70 μg), of which HPLC behavior was identical with that of **2**. The nucleoside was dissolved in a mixture of Ac_2O-d_6 (15 mg) and pyridine (40 mg), kept at room temperature for 2 h, and the product was purified by TLC on silica gel [CH_2Cl_2–MeOH (30 : 1, v/v)] to afford the acetylated nucleoside, MS *m/z* (%): 470 (M^+) (26), 268 (27), 203 (100), 142 (64). The CD spectrum of this compound was superimposable on that of **3**.

For measurement of ^{1}H-NMR spectrum of this substance, one ampoule (0.75 ml) of 100% $CDCl_3$ with 0.03% Me_4Si (Euriso-top CEA Group) was filtered through a layer of alumina 90 (Merck) (a thickness of 1 cm), which was packed on a small amount of a cotton plug in a Pasteur pipette, immediately before use. ^{1}H-NMR ($CDCl_3$) δ: 2.34 (3H, br s), 4.19 (3H, s), 4.31 (1H, dd), 4.33 (1H, dd), 4.51 (1H, ddd), 5.50 (1H, dd), 5.85 (1H, dd), 6.23 (1H, d), 7.46 (1H, br s), 7.77 (1H, s). The ^{1}H-NMR spectrum was superimposable on that obtained for **3** under identical conditions.

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