Practical Synthesis of Wybutosine, the Hypermodified Nucleoside of Yeast Phenylalanine Transfer Ribonucleic Acid

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An improved synthesis of 3-β-D-ribofuranosylwybutine (2) has been achieved by the Wittig reaction between 4,6-dimethyl-9-oxo-3-[2,3,5-tris-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-4,9-dihydro-3*H*-imidazo[1,2-*a*]-purine-7-carbaldehyde (8) and the phosphorane derived from (*R*)-2-[(methoxycarbonyl)amino]-3-(triphenylphosphonio)propanoate (9), followed successively by methylation, hydrogenation, and deprotection. On the other hand, the minor nucleoside wybutosine of yeast tRNA^{Phe} was isolated on a scale of 80 μg by partial digestion of unfractionated tRNA (1 g) with nuclease P_1, followed successively by reversed-phase column chromatography, complete digestion with nuclease P_1/alkaline phosphatase, and reversed-phase HPLC. Comparison of this nucleoside with 2 has unambiguously established that the structure of wybutosine is (α*S*)-α-[(methoxycarbonyl)-amino]-4,6-dimethyl-9-oxo-3-β-D-ribofuranosyl-4,9-dihydro-3*H*-imidazo[1,2-*a*]purine-7-butanoic acid methyl ester (2).

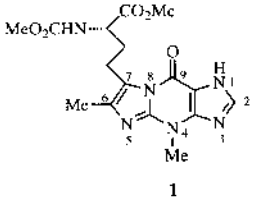
Key words wybutosine; minor nucleoside; yeast tRNA^{Phe}; fluorescent nucleoside; condensed tricyclic nucleoside; ^{1}H-NMR

Wybutosine was isolated in 1968 from the next position to the 3′-end of the anticodon of tRNA^{Phe} of baker's[1] or brewer's yeast.[2] On treatment with 0.1 M ammonium formate (pH 2.9) at 37 °C, wybutosine releases the fluorescent base wybutine (**1**),[2] whose two-dimensional structure has been elucidated by Nakanishi's and Zachau's groups.[3] The (*S*)-configuration for wybutine has been established by comparison of its circular dichroism (CD) spectrum with that of **1** obtained by our chiral synthesis.[4] Although Blobstein *et al.* isolated wybutosine on a scale of 90 μg by digestion of tRNA^{Phe} with pancreatic RNase followed successively by chromatography on Whatman DE-52, digestion with snake venom phosphodiesterase, chromatography on Sephadex G-25, digestion with RNase T_2, column chromatography on Sephadex G-25 and then on Bio Gel P-2, and TLC on cellulose,[5] rigorous identification of the position of glycosylation and the structure of the sugar moiety was difficult at that time. Nineteen years later, we synthesized 3-β-D-ribofuranosylwybutine (**2**),[6] which has been accepted as the most probable structure for wybutosine. However, any groups no longer kept a sample of wybutosine to be compared with **2**. We report here a convenient procedure for isolation of wybutosine from unfractionated tRNA of baker's yeast and identification of its structure with **2**, of which improved synthesis is also described. A preliminary communication of this work has been published.[7,8]

Results and Discussion

Improved Synthesis of 2 The first synthesis of **2** has been attained through the Heck reaction between (*S*)-2-[(methoxycarbonyl)amino]-3-butenoic acid and 7-iodo-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)wye (**3**). The Heck reaction, however, was accompanied by partial epimerization at the amino acid moiety, and **2** was obtained in only a small quantity after catalytic hydrogenation followed successively by repeated HPLC, methylation, and deprotection (Chart 1).[6] On the other hand, the first chiral synthesis of **1** has been accomplished through the Wittig reaction of 1-benzyl-7-formylwye employing (*R*)-[2-carboxy-2-[(methoxycarbonyl)-amino]ethyl]triphenylphosphonium chloride followed successively by methylation, hydrogenation, and debenzylation.[4] Unfortunately, the Wittig reaction at the nucleoside level using 7-formyl-3-(2,3,5-tri-*O*-benzyl-β-D-ribofuranosyl)wye or 7-formyl-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)wye did not afford the desired olefin, probably owing to the acidity and/or electrophilicity of the protecting groups.[7]

In the present study, we carried out again the Wittig reaction using the inner salt **9**, which had been shown to be a better reagent than the phosphonium chloride.[9,10] The aldehyde we selected was the silyl ether **8**. Although the labile glycosyl bond of 2′,3′,5′-*O*-tris(*tert*-butyldimethylsilyl)wyosine (**7**)[11] was tolerant under the conditions of the Vilsmeier reaction at −5 °C for 75 min, an aldehyde of bis(*tert*-butyldimethylsilyl) compound **11** was obtained in 21% yield along with **8** (23%) and **7** (19%). Treatment of **11** with MeONa in MeOH gave compound **12**, of which ^{1}H-NMR spectrum [$(CD_3)_2SO$] was characteristic of a primary alcohol, confirming the correctness of structure **11**. The Vilsmeier reaction of **7** was then conducted at −30 °C for 70 min to provide **8** as a colorless glass in 64% yield with 26% recovery of **7**. The Wittig reaction of **8** employing the inner salt **9** was performed according to the reported procedure[10] and subsequent methylation with Me_3SiCHN_2 afforded the olefin **10** in 38% yield. Catalytic hydrogenation using Pd–C followed by desilylation by treatment with Bu_4NF afforded crude **2**, which was shown by HPLC analysis to be contaminated by the epimer **15** to an extent of 1%. Purification of **2** was easily accomplished by reversed-phase HPLC, and **2** was obtained



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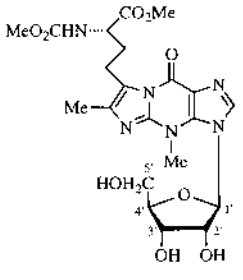

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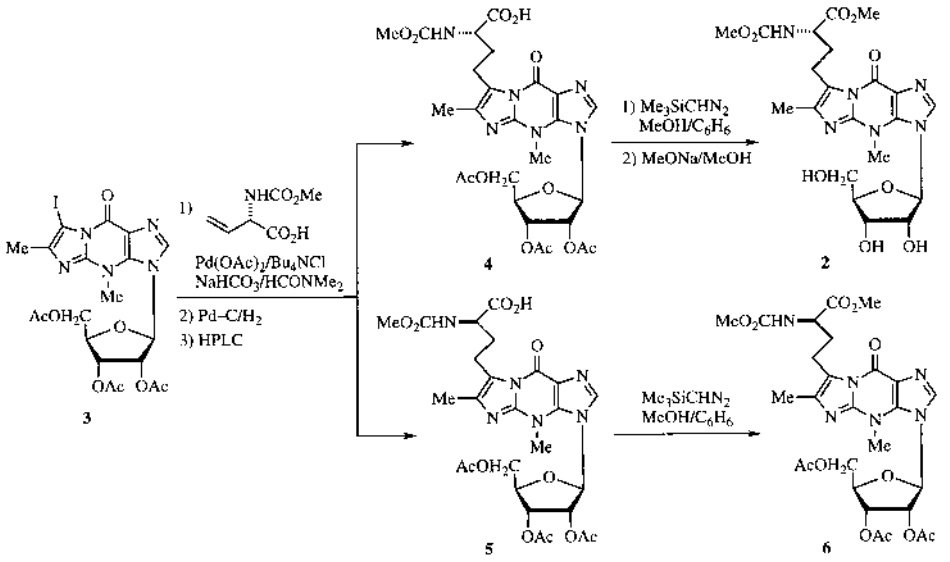


Chart 1

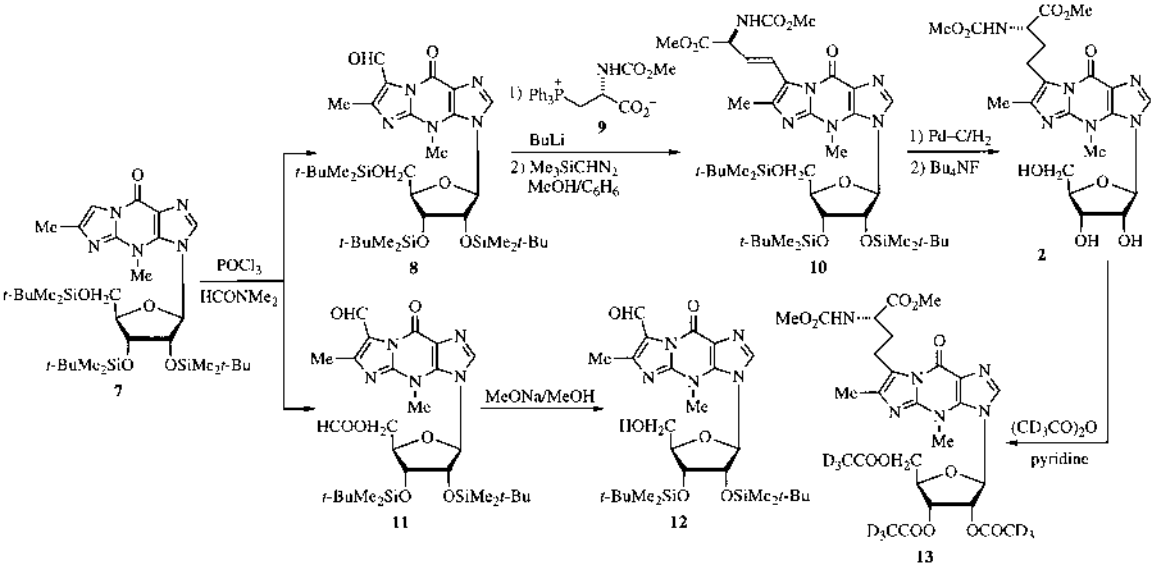
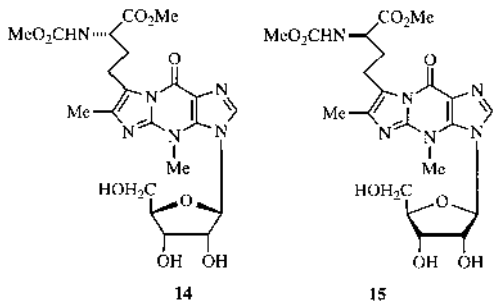


Chart 2

as a colorless glass in 63% overall yield based on **10**.

Isolation and Identification of Wybutosine We have already reported large-scale isolation of the minor nucleoside wyosine from unfractionated torula yeast tRNA and determined its structure by comparison of the ^{1}H-NMR spectrum of its triacetate-d_9[12] with that of chemically synthesized 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.[13] According to this procedure unfractionated tRNA (1 g) obtained from dry yeast (*Saccharomyces cerevisiae*) was treated with an insufficient amount of nuclease P_1 (490 units) at pH 5.3 and 50 °C for 3 h. The hydrolysate was then purified by reversed-phase column chromatography. The lipophilic fraction that was concentrated was further digested with nuclease P_1 (1500 units) (at pH 5.3 and 50 °C for 7 h) and then with alkaline phosphatase. The mixture of nucleosides thus obtained was purified by reversed-phase HPLC, providing wybutosine (1 A_{310} unit, *ca.* 80 μg), of which HPLC behavior was identical with



that of **2** but different from that of the epimer **15**. Thus the 3-β-L-ribofuranosyl structure **14** was ruled out for the structure of wybutosine, since **15** is the enantiomer of **14**. Wybutosine was then converted into the triacetate-d_9, which was identical with the triacetate-d_9 **13** prepared from **2** on the basis of their superimposable MS and ^{1}H-NMR spectra. The structure of wybutosine was hereby determined to be (αS)-α-[(methoxy-

carbonyl)amino]-4,6-dimethyl-9-oxo-3-β-D-ribofuranosyl-4,9-dihydro-3*H*-imidazo[1,2-*a*]purine-7-butanoic acid methyl ester (**2**).

Experimental

General Notes Spectra reported herein were recorded on a JEOL JMS-SX102A mass spectrometer, a JEOL JNM-GSX-500 or a JEOL JNM-EX-270 NMR spectrometer (measured at 25 °C with Me_4Si as an internal standard). $CDCl_3$ for measurements of small samples of **13** was treated with alumina according to the reported procedure.[13] MS measurements were performed by Dr. M. Takani and her associates at Kanazawa University. The optical rotation was measured with a Horiba SEPA-300 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, and a Chromatocorder 21 integrator or a Waters 6000A pump, a U6K injector, and a model 440 absorbance detector. The following abbreviations are used: br=broad, d=doublet, dd=doublet-of-doublets, ddd=doublet-of-doublets-of-doublets, m=multiplet, s=singlet.

4,6-Dimethyl-9-oxo-3-[2,3,5-tris-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-4,9-dihydro-3*H*-imidazo[1,2-*a*]purine-7-carbaldehyde (8) i) Reaction at −5 °C: A solution of $POCl_3$ (0.1 ml) in Me_2NCHO (0.5 ml) was added to a solution of **7**[11] (62 mg, 0.091 mmol) in Me_2NCHO (1 ml) at −5 °C, and the mixture was stirred at that temperature for 75 min. The reaction mixture was extracted with CH_2Cl_2 (3×5 ml) after addition of saturated aqueous $NaHCO_3$ (5 ml). The organic layers were combined, dried over $MgSO_4$, and concentrated *in vacuo.* The oily residue was purified by flash chromatography [hexane–AcOEt (3 : 2, v/v)]. Compound **8** (15 mg, 23%) was obtained from the first fraction as a colorless glass, $[\alpha]_D^{34}$ −38.9° (*c*=1.00, MeOH). MS *m/z*: 705.3761 (Calcd for $C_{33}H_{59}N_5O_6Si_3$: 705.3773). ^{1}H-NMR ($CDCl_3$) δ: −0.28, −0.01, 0.13, (3H each), 0.15 (6H), 0.16 (3H) (s, three $SiMe_2$'s), 0.73, 0.95, 0.96 (9H each, s, three *tert*-Bu's), 2.67 [3H, s, C(6)-Me], 3.80 (dd, *J*=11.5, 2 Hz), 3.89 (dd, *J*=11.5, 2.9 Hz) [1H each, C(5′)-H_2], 4.14 [1H, dd, *J*=2, 2.9 Hz, C(4′)-H], 4.21 [1H, d, *J*=4.4 Hz, C(3′)-H], 4.22 (3H, s, NMe), 4.40 [1H, dd, *J*=4.4, 7.8 Hz, C(2′)-H], 6.26 [1H, d, *J*=7.8 Hz, C(1′)-H], 8.04 [1H, s, C(2)-H], 10.97 (1H, s, CHO). The starting material **7** (12 mg, 19%) was recovered from the second fraction as a colorless solid, mp 112—120.5 °C. 3-[5-*O*-Formyl-2,3-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-4,6-dimethyl-9-oxo-4,9-dihydro-3*H*-imidazo[1,2-*a*]purine-7-carbaldehyde (**11**) (12 mg, 21%) was obtained from the third fraction as a colorless foam, MS *m/z*: 619 (M^+). ^{1}H-NMR ($CDCl_3$) δ: −0.19, −0.01, 0.15, 0.16 (3H each, s, two $SiMe_2$'s), 0.76, 0.96 (9H each, s, two *tert*-Bu's), 2.66 [3H, s, C(6)-Me], 4.20—4.45 [5H, m, C(2′)-H, C(3′)-H, C(4′)-H, C(5′)-H_2], 4.24 (3H, s, NMe), 6.26 [1H, d, *J*=7 Hz, C(1′)-H], 7.89 (1H, s, HCOO), 8.16 [1H, s, C(2)-H], 10.94 [1H, s, C(7)-CHO].

ii) Reaction at −30 °C: A solution of $POCl_3$ (5 ml) in Me_2NCHO (25 ml) was added to a solution of **7**[11] (3.63 g, 5.35 mmol) in Me_2NCHO (21 ml) at −30 °C over a period of 10 min under N_2, and the mixture was stirred at −30 °C for a further 1 h. The reaction mixture was poured onto a mixture of ice (50 ml) and saturated aqueous $NaHCO_3$ (350 ml), and the whole was extracted with AcOEt (2×100 ml). The organic layers were combined, washed with H_2O (3×400 ml), dried over $MgSO_4$, and concentrated *in vacuo.* The oily residue was purified by flash chromatography [hexane–AcOEt (3 : 2, v/v)]. Compound **8** (2.22 g) was obtained from the first fraction. A mixture (825 mg) of **8** and **7** was obtained from the second fraction. Compound **7** (354 mg) was recovered from the third fraction. The mixture of **8** and **7** was further purified by flash chromatography and preparative TLC on silica gel [hexane–AcOEt (3 : 2, v/v)], providing **8** (208 mg, the total yield was 64%) and **7** (581 mg, the total recovery was 26%).

3-[2,3-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-4,6-dimethyl-9-oxo-4,9-dihydro-3*H*-imidazo[1,2-*a*]purine-7-carbaldehyde (12) A solution of **11** (20 mg, 0.032 mmol) in 0.05 M MeONa–MeOH (1.6 ml) was stirred at 0 °C for 5 min, and cold aqueous 0.05 M NaH_2PO_4 (3.2 ml) was added at once. The mixture was extracted with CH_2Cl_2 (2×5 ml). The organic layers were combined, washed with H_2O (20 ml), dried over $MgSO_4$, and concentrated *in vacuo.* The residue was purified by preparative TLC on silica gel [CH_2Cl_2–MeOH (30 : 1, v/v)], providing **12** (9 mg, 47%) as a colorless glass, MS *m/z*: 591 (M^+). ^{1}H-NMR ($CDCl_3$) δ: −0.26, 0.00, 0.15, 0.16 (3H each, s, two $SiMe_2$'s), 0.75, 0.96 (9H each, s, two *tert*-Bu's), 2.67 [3H, s, C(6)-Me], 3.93 (dd, *J*=12, 1 Hz), 4.09 (dd, *J*=12, 2 Hz) [1H each, C(5′)-H_2], 4.19 [1H, dd, *J*=1, 2 Hz, C(4′)-H], 4.24 (3H, s, NMe), 4.37 [1H, d, *J*=4 Hz, C(3′)-H], 4.74 (1H, br, OH), 4.81 [1H, dd, *J*=4, 7 Hz, C(2′)-H], 6.28 [1H, d, *J*=7 Hz, C(1′)-H], 8.54 [1H, s, C(2)-H], 10.94 (1H, s, CHO). ^{1}H-NMR [$(CD_3)_2SO$] δ: −0.28, −0.01, 0.13, 0.14 (3H each, s, two $SiMe_2$'s), 0.73, 0.92 (9H each, s, two *tert*-Bu's), 2.55 [3H, s, C(6)-Me], 3.65 (ddd, *J*=12, 3, 5.5 Hz), 3.75 (ddd, *J*=12, 4, 4.5 Hz) [1H each, C(5′)-H_2], 4.04 [1H, ddd, *J*=3, 4, 2 Hz, C(4′)-H], 4.15 (3H, s, NMe), 4.30 [1H, dd, *J*=2, 4 Hz, C(3′)-H], 4.57 [1H, dd, *J*=4, 7 Hz, C(2′)-H], 5.29 (1H, dd, *J*=4.5, 5.5 Hz, $CH_2O\underline{H}$), 6.26 [1H, d, *J*=7 Hz, C(1′)-H], 8.37 [1H, s, C(2)-H], 10.76 (1H, s, CHO).

[*S*-(*E*)]-4-[4,6-Dimethyl-9-oxo-3-[2,3,5-tris-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-4,9-dihydro-3*H*-imidazo[1,2-*a*]purin-7-yl]-2-[(methoxycarbonyl)amino]-3-butenoic Acid Methyl Ester (10) The phosphonium salt **9**[9] (*ca.* 2.4 g) was dried three times by coevaporation with $CHCl_3$–benzene (20 ml each) and then dried over P_2O_5 at 2 mmHg and 80 °C for 16 h. Dried **9** (2.02 g, 4.96 mmol) was dissolved in a mixture of $(Me_2N)_3PO$ (29 ml) and tetrahydrofuran (THF) (82 ml) under N_2. A 1.57 M solution of BuLi in hexane (6.3 ml, 9.89 mmol) was added dropwise to the stirred solution of **9** at −70 °C under N_2 over a period of 15 min, and the whole was stirred for a further 20 min. A solution of **8** (3.10 g, 4.39 mmol) in THF (45 ml) was added to the mixture over a period of 10 min at −70 °C, and the whole was stirred at that temperature for a further 1 h. The resulting mixture was warmed to 0 °C over a period of 30 min with stirring, neutralized with 10% aqueous H_3PO_4 (6 ml), and concentrated *in vacuo* to one fifth the initial volume. H_2O (200 ml) and saturated aqueous NaCl (50 ml) were added to the residue, and the mixture was extracted with AcOEt (100 ml and then 2×50 ml). The organic layers were combined, washed successively with saturated aqueous NaCl and H_2O (300 ml each), dried over $MgSO_4$, and concentrated *in vacuo*, leaving a slightly yellow foam. This was purified by flash chromatography [hexane–AcOEt (3 : 2, v/v), $CHCl_3$–MeOH (10 : 1, v/v), and then $CHCl_3$–MeOH (3 : 1, v/v)]. The starting material (515 mg, 17%) was recovered from the hexane–AcOEt fraction. Crude carboxylic acid obtained from $CHCl_3$–MeOH (3 : 1, v/v) fraction as a yellow foam was suspended in H_2O (30 ml), brought to pH 3 with 10% aqueous H_3PO_4, and extracted with AcOEt (3×30 ml). The organic layers were combined, dried over $MgSO_4$, and concentrated *in vacuo.* The residue (1.67 g) was dissolved in a mixture of MeOH (10 ml) and benzene (40 ml), and a 2 M solution of Me_3SiCHN_2 in hexane (2 ml, 4 mmol) was added. The resulting yellow solution was concentrated *in vacuo*, and the residual yellow foam was purified by flash chromatography [hexane–AcOEt (2 : 3, v/v)] to give **10** (1.41 g, 38%) as a yellow foam, $[\alpha]_D^{17}$ +16.4° (*c*=1.00, $CHCl_3$). FAB-MS *m/z*: 849.4424 (MH^+) (Calcd for $C_{39}H_{69}N_6O_9Si_3$: 849.4434). ^{1}H-NMR ($CDCl_3$) δ: −0.29, −0.04, 0.12 (3H each), 0.137 (6H), 0.144 (3H) (s each, three $SiMe_2$'s), 0.73, 0.94, 0.95 (9H each, s, three *tert*-Bu's), 2.37 [3H, s, C(6)-Me], 3.73 (3H, s, NCO_2Me), 3.78 [1H, dd, *J*=11.5, 2 Hz, one of C(5′)-H_2], 3.82 (3H, s, CCO_2Me), 3.87 [1H, dd, *J*=11.5, 2.9 Hz, one of C(5′)-H_2], 4.10 (3H, s, NMe), 4.11 [1H, dd, *J*=2, 2.9 Hz, C(4′)-H], 4.19 [1H, d, *J*=4 Hz, C(3′)-H], 4.37 [1H, dd, *J*=4, 7.5 Hz, C(2′)-H], 5.12 [1H, br, C(α)-H], 5.53 (1H, br, NH), 5.85 [1H, br d, *J*=16 Hz, C(β)-H], 6.21 [1H, d, *J*=7.5 Hz, C(1′)-H], 7.74 [1H, d, *J*=16 Hz, C(γ)-H], 7.91 [1H, s, C(2)-H].

(αS)-α-[(Methoxycarbonyl)amino]-4,6-dimethyl-9-oxo-3-β-D-ribofuranosyl-4,9-dihydro-3*H*-imidazo[1,2-*a*]purine-7-butanoic Acid Methyl Ester (2) A solution of **10** (85 mg, 0.1 mmol) in MeOH (26 ml) was shaken under H_2 in the presence of 10% Pd–C (85 mg) for 3 h. The catalyst was filtered off and washed with hot MeOH (100 ml). The filtrate and washings were combined and concentrated *in vacuo* to leave (αS)-α-[(methoxycarbonyl)amino]-4,6-dimethyl-9-oxo-3-[2,3,5-tris-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-4,9-dihydro-3*H*-imidazo[1,2-*a*]purine-7-butanoic acid methyl ester (77 mg) as a colorless glass, FAB-MS *m/z*: 873 (MNa^+), 851 (MH^+). ^{1}H-NMR ($CDCl_3$) δ: −0.28, −0.03, 0.12, 0.138, 0.144, 0.15 (3H each, s, three $SiMe_2$'s), 0.74, 0.94, 0.96 (9H each, s, three *tert*-Bu's), 2.07, 2.17 [1H each, m, C(β)-H_2], 2.21 [3H, s, C(6)-Me], 2.95, 3.35 [1H each, m, C(γ)-H_2], 3.70, 3.71 (3H each, s, CCO_2Me and NCO_2Me), 3.79 (dd, *J*=11.2, 1.5 Hz), 3.87 (dd, *J*=11.2, 2.9 Hz) [1H each, C(5′)-H_2], 4.08 (3H, s, NMe), 4.11 [1H, dd, *J*=1.5, 2.9 Hz, C(4′)-H], 4.19 [1H, d, *J*=4.4 Hz, C(3′)-H], 4.32 [1H, m, C(α)-H], 4.40 [1H, dd, *J*=4.4, 7.8 Hz, C(2′)-H], 6.02 (1H, d, *J*=7.3 Hz, NH), 6.20 [1H, d, *J*=7.8 Hz, C(1′)-H], 7.92 [1H, s, C(2)-H]. A 1 M Bu_4NF solution (0.6 ml, 0.6 mmol) in THF was added to a solution of this product (60 mg) in pyridine–THF–H_2O (1 : 8 : 1, v/v) (3 ml), and the mixture was stirred at room temperature for 18 h. The resulting solution was concentrated *in vacuo.* The residue was purified by flash chromatography [CH_2Cl_2–MeOH (5 : 1, v/v)] to give a 99 : 1 mixture (32 mg) of **2** and **15**. This product was purified by HPLC [LiChrosorb RP18 (7 μm, 250×10 mm) (Merck); MeOH–H_2O (30 : 70, v/v)] in nine portions, providing **2** (25 mg, 63% overall yield based on **10**) as a colorless glass, ^{1}H-NMR [$(CD_3)_2SO$] δ: 1.86 [1H, m, one of C(β)-H_2], 2.08 [3H, s, C(6)-Me], 2.11 [1H, m, one of C(β)-H_2], 2.98, 3.11 [1H each, m, C(γ)-H_2], 3.56, 3.58 [3H each, s, overlapping with a one-proton multiplet due to one of C(5′)-H_2, NCO_2Me and CCO_2Me], 3.68 [1H, ddd, *J*=12.5, 3.4, 4.9 Hz, one of C(5′)-H_2], 3.87 [1H,

m, C(α)-H], 3.99 [1H, ddd, J=3.4, 3.4, 4.9 Hz, C(4′)-H], 4.03 (3H, s, NMe), 4.13 [1H, ddd, J=4.9, 4.9, 5.4 Hz, C(3′)-H], 4.44 [1H, ddd, J=5.4, 5.9, 4.9 Hz, C(2′)-H], 5.12 [1H, dd, J=5.4, 4.9 Hz, C(5′)-OH], 5.32 [1H, d, J=5.4 Hz, C(3′)-OH], 5.70 [1H, d, J=5.9 Hz, C(2′)-OH], 6.10 [1H, d, J=4.9 Hz, C(1′)-H], 7.35 (0.1H, br), 7.67 (0.9H, d, J=7.8 Hz) (NH), 8.21 [1H, s, C(2)-H]. This was identical (by comparison of the ^{1}H-NMR spectrum and HPLC mobility) with an authentic sample.[6)]

(αS)-α-[(Methoxycarbonyl)amino]-4,6-dimethyl-9-oxo-3-[2,3,5-tri-*O*-(acetyl-d_3)-β-D-ribofuranosyl]-4,9-dihydro-3*H*-imidazo[1,2-*a*]purine-7-butanoic Acid Methyl Ester (13) A solution of **2** (10 mg, 0.02 mmol) in a mixture of Ac_2O-d_6 (32 mg, 0.3 mmol) and pyridine (75 mg, 0.95 mmol) was kept at room temperature for 3 h and concentrated *in vacuo.* The residue was purified by preparative TLC on silica gel [CH_2Cl_2–MeOH (30 : 1, v/v)], giving **13** (10 mg, 77%) as a colorless glass, MS *m*/*z*: 643 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.05, 2.16 [1H each, m, C(β)-H_2], 2.22 [3H, s, C(6)-Me], 2.99 (ddd, J=14.6, 5.4, 10 Hz), 3.31 (ddd, J=14.6, 4.9, 10.5 Hz), [1H each, C(γ)-H_2], 3.70, 3.71 (3H each, s, CCO_2Me and NCO_2Me), 4.12 (3H, s, NMe), 4.32 [3H, m, C(α)-H and C(5′)-H_2], 4.50 [1H, ddd, J=2.5, 2.5, 3.9 Hz, C(4′)-H], 5.49 [1H, dd, J=3.9, 5.5 Hz, C(3′)-H], 5.83 [1H, dd, J=5.5, 6 Hz, C(2′)-H], 5.86 (1H, d, J=8 Hz, NH), 6.19 [1H, d, J=6 Hz, C(1′)-H], 7.73 [1H, s, C(2)-H].

(αR)-α-[(Methoxycarbonyl)amino]-4,6-dimethyl-9-oxo-3-β-D-ribofuranosyl-4,9-dihydro-3*H*-imidazo[1,2-*a*]purine-7-butanoic Acid Methyl Ester (15) A solution of **6**[6)] (7.8 mg, 0.012 mmol) in 0.1 M MeONa–MeOH (0.6 ml) was stirred at 0 °C for 5 min, and 0.1 M aqueous NaH_2PO_4 (1.2 ml) was added at once. The resulting solution was concentrated *in vacuo*, and the residue was purified by preparative TLC on silica gel [$CHCl_3$–MeOH (5 : 1, v/v)] to give **15** (5.6 mg, 90%) as a colorless glass, ^{1}H-NMR [$(CD_3)_2SO$] δ: 1.86 [1H, m, one of C(β)-H_2], 2.08 [3H, s, C(6)-Me], 2.11 [1H, m, one of C(β)-H_2], 2.96, 3.13 [1H each, m, C(γ)-H_2], 3.56, 3.58 [3H each, s, overlapping with a one-proton multiplet due to one of C(5′)-H_2, NCO_2Me and CCO_2Me], 3.69 [1H, ddd, J=12.2, 2.9, 4.9 Hz, one of C(5′)-H_2], 3.85 [1H, m, C(α)-H], 3.99 [1H, ddd, J=2.9, 4.9, 4.9 Hz, C(4′)-H], 4.03 (3H, s, NMe), 4.13 [1H, ddd, J=4.9, 4.4, 5.4 Hz, C(3′)-H], 4.44 [1H, ddd, J=4.4, 5.9, 4.9 Hz, C(2′)-H], 5.13 [1H, dd, J=4.9, 4.9 Hz, C(5′)-OH], 5.31 [1H, d, J=5.4 Hz, C(3′)-OH], 5.70 [1H, d, J=5.9 Hz, C(2′)-OH], 6.10 [1H, d, J=4.9 Hz, C(1′)-H], 7.36 (0.1H, br), 7.68 (0.9H, d, J=7.8 Hz) (NH), 8.21 [1H, s, C(2)-H].

Isolation and Identification of Wybutosine To a suspension of dry *Saccharomyces cerevisiae* (Sigma Chemical, type I) (500 g) in Tris buffer [0.02 M $(HOCH_2)_3CNH_2$–HCl (pH 7.5) containing 0.01 M $MgCl_2$] (15 l) was added 88% (v/v) aqueous phenol (7.5 l), and the mixture was shaken for 3 h. The mixture was centrifuged (8000 rpm, 10 min) at 2 °C after storage at 4 °C overnight. NaCl (394 g) and $MgCl_2 \cdot 6H_2O$ (27 g) was dissolved in the aqueous layer (13.5 l), and EtOH (13.5 l) was added to the resulting solution. The mixture was kept at 4 °C for 2 d, and the precipitate that separated was collected by decantation followed by centrifugation. The precipitate was extracted with the Tris buffer (3×250 ml). Cetyltrimethylammonium bromide (12 g) was added to the combined extracts (88500 A_{260} units), and the resulting suspension was kept at 4 °C for 3 d. The precipitate that deposited was collected by centrifugation, dissolved in the Tris buffer containing 1 M NaCl (500 ml), and precipitated again by addition of EtOH (500 ml). The reprecipitation with EtOH was repeated two more times. The precipitate thus obtained was washed successively with 67% (v/v) aqueous EtOH (containing 0.1 M NaCl–0.005 M $MgCl_2$) (300 ml), EtOH (300 ml), and Et_2O (200 ml), and dried over P_2O_5, giving crude tRNA (4.85 g). This product (3.81 g, 52000 A_{260} units) was dissolved in the Tris buffer (40 ml) and subjected to a column packed with ion exchange cellulose (Whatman DE 32) (wet volume 160 ml), which had been equilibrated with the Tris buffer. The column was washed with the Tris buffer (160 ml) followed by elution with the Tris buffer (1.5 l) containing 1 M NaCl at the rate of 400 ml per hour. EtOH (3 l) was added to the eluate and the precipitate was collected by centrifugation, washed successively with 67% (v/v) aqueous EtOH (containing 0.1 M NaCl–0.005 M $MgCl_2$), EtOH, and Et_2O (200 ml each), and dried over P_2O_5, providing tRNA (2.58 g).

This product (1 g, 17400 A_{260} units) was treated with nuclease P_1 (Yamasa) (1 mg, 490 units) in 0.02 M acetate buffer (pH 5.3, 60 ml) at 50 °C for 3 h. The hydrolysate was neutralized with 10% aqueous NaOH, concentrated *in vacuo* to 20 ml, and purified by column chromatography on Cosmosil 140C_{18}-OPN (Nacalai Tesque) (20 g) [H_2O (160 ml) and then MeOH–H_2O (30 : 70, v/v)] in two portions. The MeOH–H_2O fractions (200 ml) were combined and concentrated *in vacuo* to leave a glass (490 A_{260} units). It was dissolved in H_2O (9 ml), and a solution of nuclease P_1 (1500 units) in 0.02 M acetate buffer (pH 5.3, 11 ml) was added. The whole was kept at 50 °C for 7 h and then 2% aqueous NH_3 (0.87 ml) and *Escherichia coli* alkaline phosphatase (Takara Shuzo) (12 units) were added. The mixture (pH 9) was kept at 50 °C for 1.5 h, neutralized with 1 N aqueous HCl, and concentrated *in vacuo* to 6 ml. This was purified by HPLC [LiChrosorb RP18 (7 μm, 250×10 mm) (Merck); MeOH–H_2O (40 : 60, v/v)] in four portions, providing wybutosine (1 A_{310} unit, *ca.* 80 μg), of which HPLC behavior [LiChrosorb RP18 (7 μm, 250×4 mm) (Merck); MeOH–H_2O (30 : 70, v/v) at the rate of 0.5 ml/min] was identical with that of **2** (retention time 39.4 min) and different from that of **15** (retention time 43.0 min). The nucleoside was dissolved in a mixture of Ac_2O-d_6 (25 mg) and pyridine (55 mg). The solution was 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* (%): 643 (25), 611 (13), 376 (20), 344 (13), 268 (7), 230 (22), 216 (100), 142 (22). ^{1}H-NMR ($CDCl_3$) δ: 2.05, 2.16 (m each), 2.22 (3H, s), 2.99, 3.31 (m each), 3.70, 3.71 (3H each, s), 4.12 (3H, s), 4.32 (3H, m), 4.50 (1H, ddd), 5.49 (1H, dd), 5.83 (1H, dd), 5.86 (1H, d), 6.19 (1H, d), 7.74 (1H, s). This compound was identical (by comparison of the MS and ^{1}H-NMR spectra) with **13**.

References and Notes

1) RajBhandary U. L., Faulkner R. D., Stuart A., *J. Biol. Chem*., **243**, 575—583 (1968).
2) Thiebe R., Zachau H. G., *Eur. J. Biochem*., **5**, 546—555 (1968).
3) *a*) Nakanishi K., Furutachi N., Funamizu M., Grunberger D., Weinstein I. B., *J. Am. Chem. Soc*., **92**, 7617—7619 (1970); *b*) Funamizu M., Terahara A., Feinberg A. M., Nakanishi K., *ibid*., **93**, 6706—6708 (1971); *c*) Frihart C. R., Feinberg A. M., Nakanishi K., *J. Org. Chem*., **43**, 1644—1649 (1978); *d*) Thiebe R., Zachau H. G., Baczynskyj L., Biemann K., Sonnenbichler J., *Biochim. Biophys. Acta*, **240**, 163—169 (1971).
4) *a*) Itaya T., Mizutani A., *Tetrahedron Lett*., **26**, 347—350 (1985); *b*) Itaya T., Mizutani A., Iida T., *Chem. Pharm. Bull*., **39**, 1407—1414 (1991).
5) Blobstein S. H., Gebert R., Grunberger D., Nakanishi K., Weinstein I. B., *Arch. Biochem. Biophys*., **167**, 668—673 (1975).
6) Itaya T., Morisue M., Shimomichi M., Ozasa M., Shimizu S., Nakagawa S., *J. Chem. Soc., Perkin Trans. 1*, **1994**, 2759—2765.
7) Itaya T., Kanai T., Iida T., *Tetrahedron Lett*., **38**, 1979—1982 (1997).
8) Itaya T., Kanai T., *Tetrahedron Lett*., **40**, 8003—8006 (1999).
9) Itaya T., Iida T., Shimizu S., Mizutani A., Morisue M., Sugimoto Y., Tachinaka M., *Chem. Pharm. Bull*., **41**, 252—261 (1993).
10) Itaya T., Kanai T., *Chem. Pharm. Bull*., **46**, 1220—1224 (1998).
11) Glemarec C., Wu J.-C., Remaud G., Bazin H., Oivanen M., Lönnberg H., Chattopadhyaya J., *Tetrahedron*, **44**, 1273—1290 (1988).
12) We preferred the triacetate-d_9 to the triacetate in order to rule out a possibility that wyosine is an acetate by nature.
13) Itaya T., Kanai T., Sawada T., *Chem. Pharm. Bull*., **50**, 547—548 (2002).