Structure of Wye (Yt Base) and Wyosine (Yt) from *Torulopsis utilis* Phenylalanine Transfer Ribonucleic Acid†

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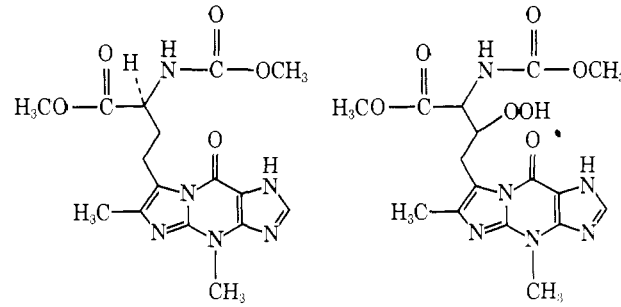
ABSTRACT: A fluorescent base wye (Yt base) was isolated from *Torulopsis utilis* $tRNA^{Phe}$. The structure was established as 4,9-dihydro-4,6-dimethyl-9-oxo-1*H*-imidazo[1,2-*a*]purine based on ultraviolet (uv), nuclear magnetic resonance (NMR), and mass spectra, and by direct comparison with synthetic material. The nucleoside, wyosine (Yt), was isolated from purified $tRNA^{Phe}$ by enzymatic degradations followed by column and thin-layer chromatographies. The structure of wyosine is proposed as 3-ribofuranosyl-4,9-dihydro-4,6-dimethyl-9-oxo-1*H*-imidazol[1,2-*a*]purine (the ribosyl group is attached to the N-9 position of guanine nucleus) on the basis of comparison with model compounds.

RajBhandary et al. (1967) first found a highly fluorescent nucleoside Y (wybutosine, yW) adjacent to the 3′ end of the anticodon in baker's yeast $tRNA^{Phe}$. Y-like fluorescent bases have also been found in the phenylalanine tRNAs from brewer's yeast (Thiebe and Zachau, 1968), wheat germ (Dudock et al., 1969), rat liver (Fink et al., 1968), and rabbit liver (Keith et al., 1973). Thiebe and Zachau (1968) reported that the Y base[1] (wybutine, Y-Wye) could be liberated by mild acid treatment from brewer's yeast $tRNA^{Phe}$, and that the Y-Wye-deficient $tRNA^{Phe}$ could be charged with phenylalanine but had lost the ability of codon recognition. Due to the strong fluorescence and the biological interest of Y-Wye, its structure has been investigated by many workers. Structures of Y-Wye from baker's yeast and O_2Y-Wye (wybutoxine; previous name: peroxy Y base) from liver and the plant *Lupinus luteus* were assigned by Nakanishi et al. (1970, 1971; Blobstein et al., 1973; Feinberg et al., 1974) as **1** and **2**, respectively; the side-chain absolute configuration of Y-Wye has been determined as L (or S) and its synthesis has also been reported (Funamizu et al., 1971).



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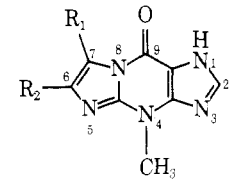
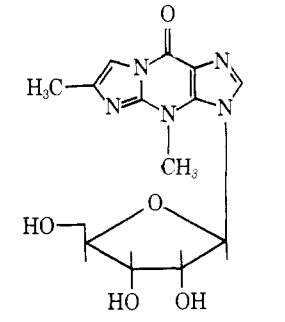


3, R_1 = H; R_2 = CH_3
5, R_1 = CH_3; R_2 = H
6, R_1 = R_2 = H



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From the studies on the nucleotide sequences in *Torulopsis utilis* $tRNA^{Phe}$ (Miyazaki and Takemura, 1968), it was supposed that a Y-like nucleoside should be present near the 3′ end of the anticodon. Indeed, a fluorescent base has been isolated from a partially purified preparation of this tRNA, to which the name Yt base (t, *Torulopsis;* present name, wye; see Table I) has been given. The corresponding nucleoside Yt (present name, wyosine) was also isolated from the anticodon region of *T. utilis* $tRNA^{Phe}$ (Takemura et al., 1974). This paper reports in detail the structural studies on wye and wyosine from *T. utilis* $tRNA^{Phe}$.

Experimental Section

Materials. BD-cellulose[1] was prepared according to the method of Gillam et al. (1967). DEAE-Sephadex A-50 and thin-layer glass plates coated with Avicel SF cellulose were purchased from Funakoshi Pharmaceutical Co., Tokyo, Japan. RNase T_1 was obtained from Sankyo Co., Ltd. RNase A, *E. coli* alkaline phosphatase, and snake venom

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[1] Abbreviations used are: $tRNA^{Phe}$, phenylalanine specific tRNA; Y, the fluorescent nucleoside found in baker's yeast $tRNA^{Phe}$; Yt, the fluorescent nucleoside found in *Torulopsis utilis* $tRNA^{Phe}$ (new names and symbols for the Y base and its nucleoside and their related compounds were recently recommended by Dr. W. Cohn, Director, Office of Biochemical Nomenclature, as shown in Table I; we will use these in this and future papers); A, adenosine; Ψ, pseudouridine; pA, 5′-adenylic acid; pΨ, 5′-pseudouridylic acid; BD-cellulose, benzoylated diethylaminoethyl-cellulose; DEAE-Sephadex, diethylaminoethyl-Sephadex; Ecteola-cellulose, ethylenechlorohydrintriethanolamine-cellulose; Me_2SO, dimethyl sulfoxide; A_{260} or A_{230} unit, an amount of material with an absorbance of 1.0 at 260 or 230 nm when dissolved in 1 ml of water and measured with a 1-cm light path; sh, shoulder; dec, decomposition.

Table I.

Previous Symbols	Base		Nucleoside		
				Symbols	
	Name	Symbols	Name	3-Letter	1-Letter
Yt, Yt^+	Wye	Wye	Wyosine	Wyo	W
Y, Y^+	Wybutine	Y-Wye	Wybutosine	Y-Wyo	yW
Peroxy Y, Yw, Yr	Wybutoxine	O_2Y-Wye	Wybutoxosine	O_2Y-Wyo	o_2yW

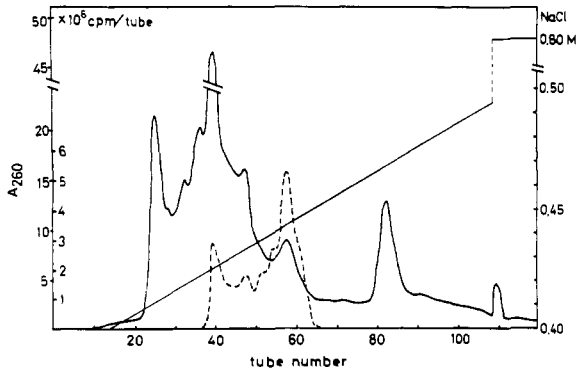


FIGURE 1: Chromatography of *T. utilis* tRNA (10 g) on a column (5.2 × 150 cm) of DEAE-Sephadex A 50. Elution was performed with a linear salt gradient formed from 12 l. of 0.40 M NaCl–0.008 M $MgCl_2$ and 12 l. of 0.52 M NaCl–0.016 M $MgCl_2$ in 0.02 M Tris-HCl (pH 7.5). Fractions of 205 ml of effluent were collected at a flow rate of 2.4 ml/min: A_{260} (—); phenylalanine acceptor activity (- - -).

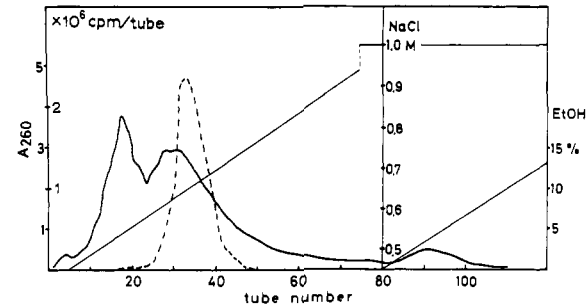


FIGURE 2: Chromatography of $tRNA^{Phe}$-rich fraction (ca. 10 000 A_{260} units) on a column (2.3 × 95 cm) of BD-cellulose. Elution was performed with a linear salt gradient formed from 4 l. of 0.45 M NaCl–0.01 M $MgCl_2$ and 4 l. of 1 M NaCl–0.01 M $MgCl_2$. The remaining tRNA was eluted with a linear gradient of 0 to 15% ethanol in 1 M NaCl (total 4 l.). Fractions of 96 ml were collected at a flow rate of 1.2 ml/min: A_{260} (—); phenylalanine acceptor activity (- - -).

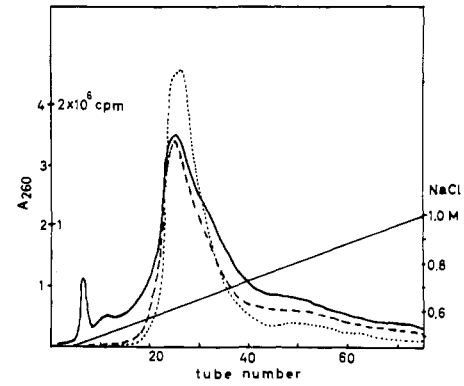


FIGURE 3: Chromatography of $tRNA^{Phe}$ on a column (0.6 × 55 cm) of BD-cellulose. Elution was performed with a linear salt gradient obtained from 150 ml of 0.45 M NaCl–0.01 M $MgCl_2$–0.01 M Tris-HCl (pH 7.5) and 150 ml of 1 M NaCl–0.01 M $MgCl_2$–0.01 M Tris-HCl (pH 7.5): A_{260} (—); phenylalanine acceptor activity (· · ·); fluorescence intensity (- - -).

phosphodiesterase were purchased from Worthington Biochemical Corp. 3-Methylguanine and 3,7-dimethylguanine were synthesized according to the methods of Townsend and Robins (1962) and Golovchinskaya et al. (1967), respectively. *T. utilis* tRNA was kindly supplied by Jūjō Paper Co., Ltd., Tokyo, Japan.

Paper and Thin-Layer Chromatography. Wye, wybutine, and wybutoxine were compared by paper chromatography on Whatman No. 1. For the comparison of natural wye with synthetic materials, Avicel SF cellulose plates were used. The solvent systems used were: (A) isobutyric acid–1 M ammonium hydroxide (5:3, v/v); (B) 2-propanol–water–concentrated ammonia (7:1:2, v/v); (C) water; (D) 4% sodium citrate; (E) 1-butanol–water–concentrated ammonia (86:14:5, v/v); (F) 2-propanol–concentrated HCl–water (70:15:15, v/v); (G) 1-butanol–acetic acid–water (4:1:2, v/v); and (H) isobutyric acid–0.5 M ammonium hydroxide (5:3, v/v).

Preparation of T. utilis $tRNA^{Phe}$. *T. utilis* tRNA (10 g) was first fractionated on a DEAE-Sephadex A-50 column (elution profile shown in Figure 1). The phenylalanine tRNA found in tubes 54 to 62 was enriched to about sevenfold. The combined fractions obtained from several runs of 10-g batches (ca. 10 000 A_{260} units) were subjected to further chromatography on a BD-cellulose column (Figure 2), and eluted with a linear concentration gradient from 0.45 to 1.0 M NaCl and further with 0–15% ethanol in 1.0 M NaCl. The *T. utilis* phenylalanine acceptor activity peak was eluted between 0.6 and 0.7 M NaCl concentrations and was not found in the ethanol fraction where baker's yeast $tRNA^{Phe}$ is eluted. Purity of the $tRNA^{Phe}$ rich fraction (tubes 29–40) was about 25% when estimated by phenylalanine acceptor activity. The above fraction (ca. 2000 A_{260} units) was charged with phenylalanine by crude phenylalanyl-tRNA synthetase from yeast and purified on a BD-cellulose column (2.2 × 7.0 cm). Phenylalanyl-$tRNA^{Phe}$ was eluted with a linear gradient of 0 to 15% ethanol in 1 M NaCl, after uncharged tRNA was washed out with 1 M NaCl. The Phe-$tRNA^{Phe}$ was deacylated and the free $tRNA^{Phe}$ (380 A_{260} units) was again purified on a BD-cellulose column. The single optical density peak coincided with phenylalanine acceptor activity and fluorescence intensity peaks as shown in Figure 3. The purity of this peak (tubes 21–37, 180 A_{260} units) was higher than 90% when estimated by the analysis of its RNase digestion products, although it was about 75% on the basis of phenylalanine acceptor activity. This preparation was used for isolation of wyosine.

Large Scale Isolation of Wye from Partially Purified $tRNA^{Phe}$. Wye was isolated according to the method of Thiebe and Zachau (1968) used for wybutine isolation from partially purified *T. utilis* $tRNA^{Phe}$. The $tRNA^{Phe}$-rich fraction (2.65 g) obtained by the first step of column chromatography on DEAE-Sephadex A-50 was dissolved in 70 ml of water and impurities were removed by extraction with chloroform (three times, each 100 ml). The aqueous solution was then acidified to pH 2.9 with hydrochloric acid and incubated at 37 °C for 4 h. After neutralization, the solution was extracted with chloroform (five times, each 100 ml). The chloroform layer was evaporated to dryness in

vacuo and the residue was purified by paper chromatography (solvent: water). The major bluish white fluorescent band with an R_f value of 0.37 and the very faint fluorescent band with an R_f value of 0.17 were detected under an ultraviolet (uv) lamp. The main band was further purified by paper chromatography with solvent F for separation of a minor uv-absorbing impurity thus yielding 25 A_{230} units (ca. 160 μg) of wye.

Isolation of Wyosine from T. utilis tRNA^{Phe}. The wyosine-containing hexanucleotide was obtained by pancreatic RNase A digestion of the largest oligonucleotide in an RNase T_1 digest of highly purified tRNA^{Phe} as described previously (Takemura et al., 1974). After removal of the 3′-terminal phosphate, the hexanucleotide (3.82 A_{260} units) was partially degraded with 25 μg of snake venom phosphodiesterase at pH 8.4 and 37 °C for 6 h. The digest was chromatographed on a DEAE-cellulose column (0.25 × 35 cm) in 7 M urea–0.01 M Tris-HCl (pH 7.5), with a NaCl concentration gradient. The 5′-mononucleotide fraction (2.1 A_{260} units), which contained pW, pA, and pΨ, was desalted, dephosphorylated with *Escherichia coli* alkaline phosphatase, and then chromatographed two dimensionally on an Avicel-SF cellulose thin-layer plate using solvent B and then solvent E. R_f values were, in solvent B: Ψ, 0.27; A, 0.52; W, 0.60; in solvent E: A, 0.23; W, 0.33. The fluorescent spot corresponding to W was scraped off and eluted with water. About 0.13 A_{260} unit of pure wyosine was obtained: λ_{max} (nm) 236, 295 (pH 4.2–12.1); A_{236}/A_{260} = 6.48; A_{295}/A_{260} = 1.56.

Spectra. Uv spectra were recorded on a Shimadzu double beam spectrophotometer (UV-200). High-resolution mass spectra were recorded on a CEC-110-B instrument with ionization potential at 70 eV. NMR spectra were obtained on a Varian 100-MHz spectrometer equipped with a Fourier transform computer. Fluorescence spectra were obtained using a Hitachi MPF-2A spectrofluorometer.

Syntheses of Related Compounds. 4,9-Dihydro-4,6-dimethyl-9-oxo-1*H*-imidazo[1,2-*a*]purine (**3**). 3-Methylguanine (500 mg) was suspended in 30 ml of 90% dimethylformamide (10% H_2O). After addition of diethylaniline (1 g) and 10% bromoacetone (25 ml, in dimethylformamide), the mixture was heated in a sealed tube at 90 °C. After 30 min, 1 h, 1.5 h, 2 h, and 3 h, 1 ml of the bromoacetone solution was added to the reaction mixture. After heating for 3.5 h the solution was allowed to stand at room temperature. The crude product (169 mg, 27.5% yield) precipitated from the solution was crystallized from water to give 119 mg of a pure material: uv $\lambda_{max}^{pH\ 1.0}$ (nm) (ϵ) 227 (37 000), 231 (37 100), 255 (5000), 284 (9700); $\lambda_{max}^{pH\ 5.8}$ 231 (33 200), 265 (5900), 306 (6500); $\lambda_{max}^{pH\ 11.0}$ 231 (36 000), 274.5 (7500), 301 (9200); pK_a (in H_2O) 3.69 ± 0.03 and 8.46 ± 0.02. Anal. Calcd for $C_9H_9N_5O$: C, 53.19; H, 4.46; N, 34.47. Found: C, 52.90; H, 4.36; N, 34.19.

4,9-Dihydro-4,7-dimethyl-9-oxo-1*H*-imidazo[1,2-*a*]purine (**5**). 3-Methylguanine (200 mg) and α-bromopropionaldehyde (500 mg) were dissolved in dimethylformamide (20 ml) and the solution was heated in a sealed tube at 100 °C for 1 h. The reaction mixture was then evaporated in vacuo until the crude product began to precipitate, and allowed to stand at room temperature to give the product (72.5 mg; yield, 29.5%). It was crystallized from hot water (about 100 ml) to give a pure material (51 mg): uv $\lambda_{max}^{pH\ 1.0}$ 229 (32 900), 232 (33 000), 251 (sh) (4200), 288 (8200); $\lambda_{max}^{pH\ 5.8}$ 232 (32 800), 256 (4800), 313 (5300); $\lambda_{max}^{pH\ 11.0}$ 233 (35 000), 264 (5300), 307 (8000). pK_a (in H_2O) 3.83 ± 0.02 and 8.68 ± 0.02. Anal. Calcd for $C_9H_9N_5O$: C, 53.19; H, 4.46; N, 34.47. Found: C, 53.05; H, 4.19; N, 33.90.

4,9-Dihydro-4-methyl-9-oxo-1*H*-imidazo[1,2-*a*]purine (**6**). 3-Methylguanine (200 mg) and chloroacetaldehyde (1 g, 30% aqueous solution) were suspended in dimethylformamide (20 ml), and the mixture was heated at 100 °C in a sealed tube for 45 min. The hot reaction mixture was filtered to remove insoluble 3-methylguanine and allowed to stand at room temperature to give crude product **6** (80 mg, yield, 35%), which was crystallized from water: uv $\lambda_{max}^{pH\ 1.0}$ 223 (33 700), 228 (33 900), 246 (4500), 283 (10 400); $\lambda_{max}^{pH\ 5.8}$ 228 (31 900), 257 (5100), 306.5 (7300); $\lambda_{max}^{pH\ 11.0}$ 229 (34 100), 269 (6000), 302 (9600); pK_a (in H_2O) 3.30 ± 0.02, 8.43 ± 0.02. Anal. Calcd for $C_8H_7N_5O$: C, 50.79; H, 3.73; N, 37.02. Found: C, 51.03; H, 3.57; N, 36.32.

3-(β-D-Ribofuranosyl)-5,9-dihydro-6-methyl-9-oxo-5*H*-imidazo[1,2-*a*]purine (**7**). Guanosine (20.0 g) was dissolved in Me_2SO (300 ml) by warming and NaH (1.7 g) was added to the solution gradually with stirring. After 2 h of stirring at room temperature, evolution of hydrogen gas stopped and the solution became clear. The solution was cooled in an ice–water bath until Me_2SO began to freeze and to this solution was added bromoacetone (9.68 g) within 30 min. After stirring for 1 h, the solution was poured onto 0.5 N KOH (1 l.) and the mixture was allowed to stand for 4 h at room temperature. It was then neutralized with acetic acid and evaporated to dryness in vacuo. The residue was dissolved in Me_2SO and applied to a silica gel column (800 g). The product was eluted with a mixture of chloroform–methanol–Me_2SO (10:1:1) and the eluates were evaporated to dryness in vacuo. Crude crystalline material was obtained by treating the residue with a small quantity of methanol. This material was further recrystallized from water to give 13.6 g (60%) of a pure material: mp 250 °C dec; NMR (Me_2SO-d_6) δ 2.27 (6-CH_3), 7.36 (7-H), and 8.16 ppm (2-H); uv $\lambda_{max}^{pH\ 1.0}$ 227 (24 900), 240 (sh) (22 300), 277 (8400), 300 (7700); $\lambda_{max}^{pH\ 7.5}$ 231 (35 500), 285 (11 700); $\lambda_{max}^{pH\ 11.3}$ 239 (32 000), 284 (sh) (5100), 308 (7000). Anal. Calcd for $C_{13}H_{15}N_5O_5$: C, 48.59; H, 4.71; N, 21.80. Found: C, 48.61; H, 4.81; N, 21.99.

3-(2′,3′-*O*-Isopropylidene-β-D-ribofuranosyl)-5,9-dihydro-6-methyl-9-oxo-5*H*-imidazo[1,2-*a*]purine. Compound **7** (3.21 g) and *p*-toluenesulfonic acid monohydrate (30 g) were dissolved in acetone (300 ml), and the solution was stirred overnight at room temperature. The reaction mixture was treated with triethylamine (20 ml) and $NaHCO_3$ (14 g), and stirred for 2 h. The mixture was filtered and washed with hot acetone (ten times, each 50 ml) and the combined filtrate and washings were evaporated to dryness in vacuo. The residue was dissolved in methanol and the solution was applied to a silica gel column (60 g). The column was eluted with a chloroform–methanol (7:1) mixture and the eluents were evaporated to dryness in vacuo. The residue was crystallized from methanol to give 2.82 g (78%) of a pure material: mp 243 °C; NMR (Me_2SO-d_6) δ 1.31, 1.52 (isopropylidene CH_3), 2.27 (6-CH_3), 7.32 (7-H), 8.10 ppm (2-H). Anal. Calcd for $C_{16}H_{19}N_5O_5$: C, 53.18; H, 5.30; N, 19.38. Found: C, 52.96; H, 5.52; N, 19.22.

5′,*N*-4-Cyclo-3-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-4,9-dihydro-6-methyl-9-oxoimidazo[1,2-*a*]purine (**8**). To a solution of an isopropylidene derivative of **7** (1.80 g) in pyridine (10 ml) was added *p*-toluenesulfonyl chloride

Table II: R_f Values of Paper Chromatography.

Compound	Solvent A	Solvent B	Solvent C
Wye	0.86	0.55	0.30
Wybutine	0.96	0.71	0.52
Wybutoxine	0.78	0.67	0.52

(1.05 g) and the mixture was allowed to stand overnight at room temperature. The solution was added slowly to ice-water (100 ml). The mixture was stirred and the precipitated solid was isolated by filtration, washed with water, and dried to give the crude tosylate (2.35 g). It was suspended in dioxane (50 ml) and refluxed for 3 h. After cooling, the precipitate was collected by filtration, dissolved in cold water (20 ml), and neutralized with concentrated ammonia to give the crude product. The pure material (1.28 g, 74.7%) was obtained by recrystallization from ethanol: mp 292.5 °C; NMR ($CDCl_3$) δ 1.35, 1.55 (isopropylidene CH_3), 2.26 (6-CH_3), 7.20 (7-H), and 7.55 (2-H) ppm; uv $\lambda_{max}^{pH\ 0.91}$ 231 (36 500), 277 (13 800); $\lambda_{max}^{pH\ 6.50}$ 236 (34 300), 295 (8900). Anal. Calcd for $C_{16}H_{17}N_5O$: C, 55.97; H, 4.99; N, 20.40. Found: C, 55.95; H, 4.85; N, 20.51.

Acid Hydrolysis of Cyclonucleoside **8** to **9**. Compound **8** (ca. 10 mg) was dissolved in 0.5 N HCl (0.5 ml) and heated at 90 °C. The reaction mixture was checked on a thin-layer plate (silica gel; solvent, chloroform–methanol, 5:1). As the reaction proceeded, the spot of **8** (R_f 0.78) was diminished, while a new spot (R_f 0.11) appeared. After 2 h only one spot (R_f 0.11) was detected. Uv spectra of this reaction mixture were: $\lambda_{max}^{pH\ 2}$ 228, 233 (sh), 283; $\lambda_{max}^{pH\ 7}$ 231, 268, 303; $\lambda_{max}^{pH\ 11}$ 232, 275, 300.

3-(β-D-Ribofuranosyl)-5,9-dihydro-5,6-dimethyl-9-oxoimidazo[1,2-*a*]purine (**10**). Compound **7** (200 mg) was dissolved in Me_2SO–methanol (1:1) solution (20 ml) and the solution was treated with ethereal diazomethane (ca. 50–60 ml) until the yellow color of the solution persisted. After standing overnight, the solution was evaporated to dryness in vacuo and the residue was crystallized by addition of methanol: yield, 185 mg (89%); mp 247 °C dec., NMR (Me_2SO-d_6) δ 2.31 (6-CH_3), 3.57 (5-CH_3), 7.43 (7-H), and 8.12 ppm (2-H); uv $\lambda_{max}^{pH\ 6.6}$ 232.5 (34 000), 289 (11 200); $\lambda_{max}^{pH\ 0.95}$ 228 (22 400), 242 (sh) (19 300), 279 (7900), 305 (6800). Anal. Calcd for $C_{14}H_{17}N_5O_5$: C, 50.14; H, 5.11; N, 20.89. Found: C, 50.23; H, 4.97; N, 20.88.

5,9-Dihydro-5,6-dimethyl-9-oxo-1*H*-imidazo[1,2-*a*]purine (**11**). Compound **10** (168 mg) was dissolved in 6 N HCl (5 ml), and the solution was heated in a sealed tube at 80 °C for 6 h. After filtration and active charcoal treatment, the product was crystallized by addition of concentrated ammonia: yield, 84 mg; mp 235 °C dec; NMR (1 N NaOD) δ 2.05 (6-CH_3), 3.42 (5-CH_3), 7.10 (7-H), and 8.23 ppm (2-H); uv $\lambda_{max}^{pH\ 6.5}$ 230, 293; $\lambda_{max}^{pH\ 2.0}$ 223, 235 (sh), 273, 302.5; $\lambda_{max}^{pH\ 10.9}$ 234, 267 (sh), 300. Anal. Calcd for $C_9H_9N_5O$: C, 53.19; H, 4.46; N, 34.47. Found: C, 52.10; H, 4.34; N, 34.52.

4,9-Dihydro-1,4,6-trimethyl-9-oxoimidazo[1,2-*a*]purine (**12**). 3,7-Dimethylguanine (1.79 g) and K_2CO_3 (2.76 g) were suspended in Me_2SO (10 ml), and to this suspension was added bromoacetone (2.74 g) at room temperature with stirring. The stirring was continued for 36 h. After the reaction was completed, water (10 ml) was added and the mixture evaporated to dryness in vacuo. The residue was treated with water (5–7 ml) and extracted with chloroform (three times, each 50 ml). The chloroform layer was concentrated to dryness in vacuo and the residue was crystallized from water: yield, 1.63 g (75%); mp 218 °C; NMR ($CDCl_3$) δ 2.33 (6-CH_3), 3.90 (N-CH_3), 4.18 (N-CH_3), 7.35 (7-H), and 7.61 ppm (2-H); uv $\lambda_{max}^{pH\ 2.42}$ 230 (sh) (34 000), 234 (34 400), 288 (8200); $\lambda_{max}^{pH\ 5.44}$ 233 (30 000), 265 (5300), 311 (6100). Anal. Calcd for $C_{10}H_{11}N_5O$: C, 55.29; H, 5.10; N, 32.24. Found: C, 55.12; H, 5.08; N, 32.38.

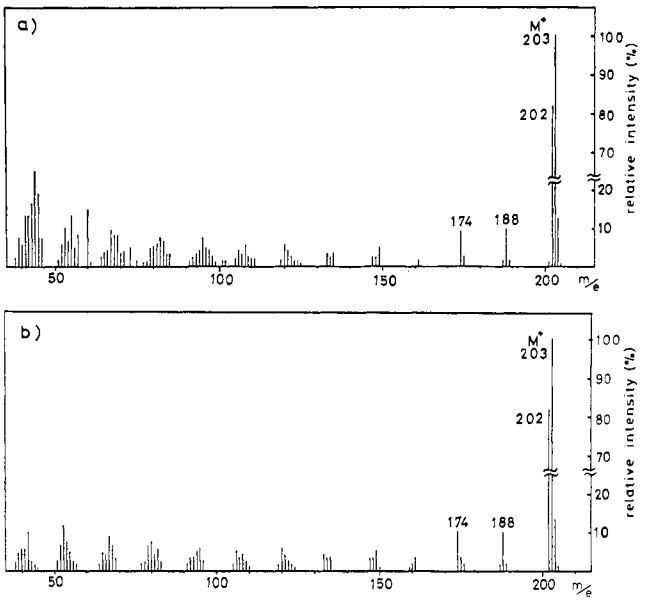


FIGURE 4: Mass spectra of (a) wye and (b) compound **3** at 70 eV.

1-(D-Ribofuranosyl)-4,9-dihydro-4,6-dimethyl-9-oxoimidazo[1,2-*a*]purine (**13**). To a fused mixture of **3** (0.8 g) and tetra-*O*-acetyl-β-D-ribofuranose (8.0 g) was added *p*-toluenesulfonic acid (400 mg) and the mixture was heated at 160 °C for 35 min. The reaction mixture was diluted with ethanol (30 ml) and allowed to stand overnight. It was filtered and the filtrate was made alkaline with ammonia and heated at 90 °C for 20 min. The solution was then evaporated to dryness and the residue was dissolved in water (50 ml), acidified with formic acid, and chromatographed successively through: (i) a Florisil column (200 ml) using water–3 M ammonium hydroxide gradient and 3 M ammonium hydroxide–3 M ammonium hydroxide/30% acetone gradient elution; (ii) an Ecteola-cellulose column using water as the solvent; and (iii) a Sephadex G-25 column using water as solvent. The fluorescent eluents were evaporated to dryness in vacuo and the residue was crystallized from aqueous ethanol to give the pure product (83 mg): mp 236–237 °C; uv $\lambda_{max}^{pH\ 1.0}$ 230 (30 300), 234 (31 100), 253 (sh) (4400), 288 (7700); $\lambda_{max}^{pH\ 7.0}$ 232 (25 800), 263 (4700), 313 (5400); pK_a (in H_2O) 3.40 ± 0.02. Anal. Calcd for $C_{14}H_{17}N_5O_5$: C, 50.14; H, 5.11; N, 20.59. Found: C, 50.17; H, 5.22; N, 20.37.

Results and Discussion

Structure of Wye. Wye is different from wybutine; this was confirmed by chromatographic comparison of these compounds (Table II). The mass spectrum of wye showed considerably high intensity peaks at *m/e* 203 (M), 202 (M − H), 188 (M − CH_3), and 174 (M − CHO) (Figure 4a). High-resolution mass spectra gave the following values: *m/e*

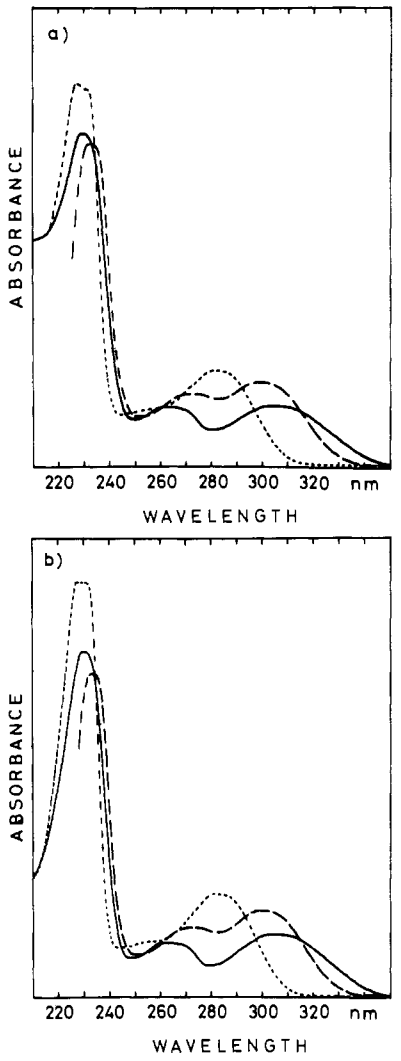


FIGURE 5: Uv spectra of (a) wye and (b) compound **3** in H_2O (—); 0.1 N HCl (···); 0.1 N NaOH (- - -).

203.0802 (M calculated for $C_9H_9N_5O$: 203.0807), 202.0730 ($C_9H_8N_5O$: 202.0729), 188.0590 ($C_8H_6N_5O$: 188.0572), and 174.0767 ($C_8H_8N_5$: 174.0780). The spectrum suggests that wye has the molecular formula $C_9H_9N_5O$ and has a very stable nucleus with carbonyl and methyl groups. Uv spectra of wye at acid, neutral, and basic pH values are highly characteristic and closely resemble those of the wybutine (Thiebe and Zachau, 1968), except that the maximum wavelengths of the former are slightly blue-shifted as compared with those of the latter (Figure 5a), suggesting that wye and wybutine have the same chromophore but different side chains. Wye has two pK_a values, i.e. 3.66 ± 0.05 and 8.52 ± 0.10, measured by the uv method. The fluorescence excitation spectrum of wye showed a maximum at 315 nm (Figure 6a) and the emission spectrum excited at 315 nm showed a maximum at 435 nm in water (Figure 6b). The NMR spectrum of wye measured in D_2O showed signals at δ 2.24 (3 H, d, J = 1 Hz), 3.82 (3 H, s), 7.32 (1 H, q, J = 1 Hz), and 8.07 ppm (1 H, s), which are assignable to an aromatic methyl, an *N*-methyl, and two aromatic protons, respectively. The small coupling (ca. 1 Hz) appeared between the signals at 2.24 ppm (methyl group) and the 7.32 ppm (1 proton) is attributable to a long-range spin–spin coupling between the methyl and the proton in the CH_3—C=C—H grouping. The above data

Table III: R_f Values of Avicel SF Cellulose Thin-Layer Chromatography.

	Solvent					
Compd	B	G	H	F	C	D
Wye	0.60	0.68	0.80	0.26	0.21	0.12
3	0.60	0.68	0.80	0.26	0.21	0.12
5	0.67	0.70	0.82	0.28	0.15	0.11
6	0.62	0.64	0.77	0.19	0.27	0.18

Table IV: Chemical Shifts (ppm) in NMR Spectra of Wye and Compound **3** in D_2O.[a]

	6-CH_3	4-CH_3	7-H	2-H
Wye	2.24	3.82	7.32	8.07
3	2.26	3.82	7.30	8.09

[a] 1000-scan Fourier transform NMR.

coupled with consideration of the proposed structure of wybutine by Nakanishi et al. (1970) strongly suggested that the structure of wye was **3** or **5**.

We therefore carried out the synthesis of two isomeric compounds **3** and **5** having the *C*-methyl group at C_6 and C_7, respectively, and the *N*-methyl group at N_4 (structure assignment, vide infra). R_f values of compound **3** using several solvent systems were identical with those of wye (Table III). Mass, uv, and NMR spectra and pK_a values of wye were all in full agreement with those of compound **3** (Figures 4 and 5 and Table IV). Hence, we conclude that the structure of wye is represented by **3**.

Recently, Kreishman et al. (1974) proposed structure **11** rather than **3** for wye, as judged from NMR spectral data on a wyosine-containing hexanucleotide. We also synthesized compound **11**, but the uv data differed from those for natural wye, and thus structure **11** can be discarded.

Structure of Wyosine. The nucleoside wyosine (previously Yt) was isolated by enzymatic degradations followed by chromatography at neutral or moderately alkaline pH to avoid cleavage of the glycosidic linkage. Uv spectra of wyosine thus obtained were unchanged at various pH values ranging from 4.3 to 12.1 (Figure 7; Takemura et al., 1974). Three model nucleosides, **8, 10,** and **13,** were synthesized in order to determine the structure of wyosine. The model compound **8** was easily hydrolyzed to compound **9** by relatively mild acid treatment (in 0.5 N HCl, at 100 °C, for 50 min), whereas compound **10** was stable under this condition (no change in 0.5 N HCl, at 100 °C for 5 h). The uv spectrum of **8** is almost identical with that of wyosine, while compound **10** shows a different spectrum from that of wyosine as shown in Figure 7. The model compound **13,** having the N-1 ribosyl structure, also shows a different uv spectrum ($\lambda_{max}^{pH\ 7.0}$ 232, 263, and 313 nm) from that of wyosine. These data indicate that the ribosyl residue is attached to the N-3 position as in structure **4;** the same point of attachment for the ribosyl moiety has been suggested recently for wybutosine (Blobstein et al., 1975).

Synthesis and Structure Assignment of the Related Compounds. Condensation of 3-methylguanine and bromoacetone gave the fluorescent compound **3,** whereas isomeric compound **5** was produced by condensation of 3-methylguanine and α-bromopropionaldehyde. Compound **6,** having no olefinic *C*-methyl group, was also synthesized by

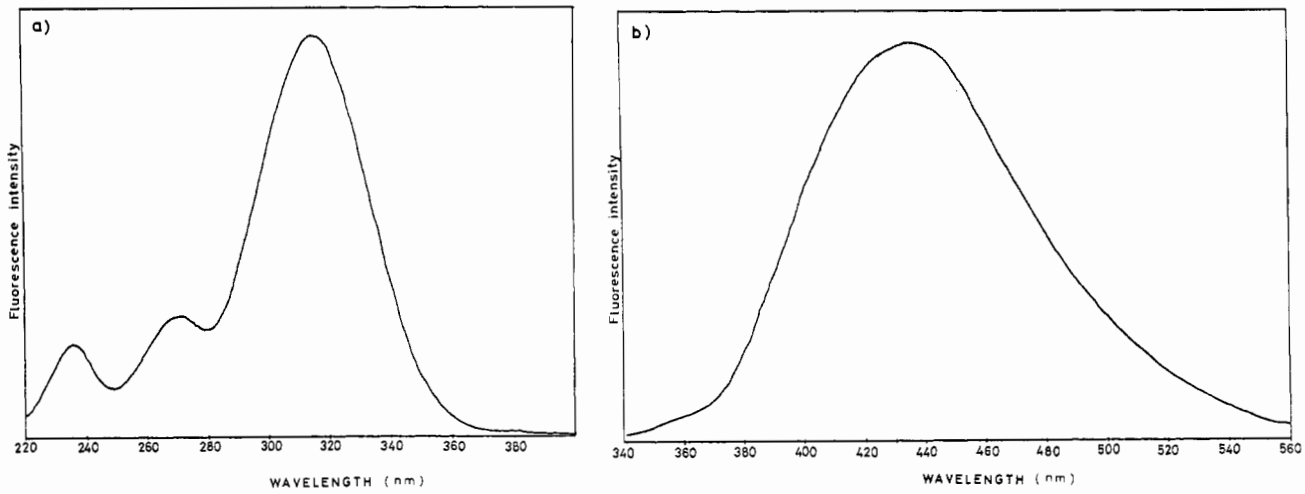


FIGURE 6: Fluorescence (a) excitation spectrum (emission at 435 nm) and (b) emission spectrum (excitation at 315 nm) of wye in water.

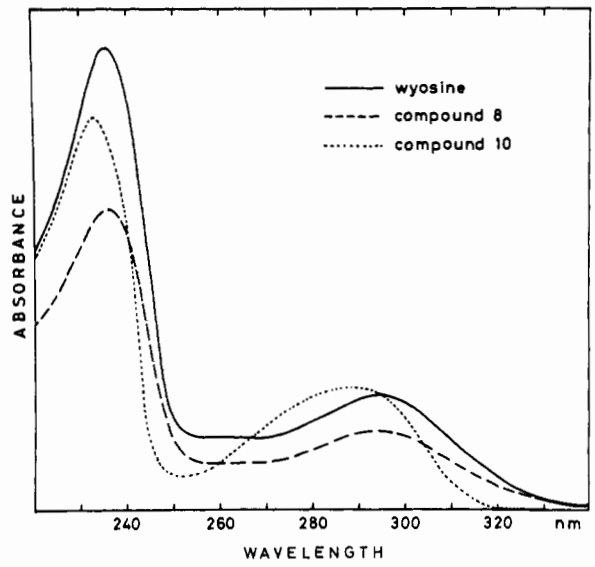
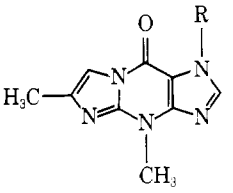


FIGURE 7: Comparison of uv spectra of wyosine, compound **8**, and compound **10**. Uv spectrum of each compound is identical between pH 4.3 and 12.1.

condensation of 3-methylguanine and chloroacetaldehyde. The position of attachment of the *C*-methyl group in compounds **3** and **5** was assigned from the NMR data measured in 1 N DCl–D_2O. Compound **3** shows the *C*-methyl signal (2.48 ppm) at higher magnetic field than the corresponding signal (2.72 ppm) of compound **5**, whereas the aromatic proton signal which is spin coupled to the *C*-methyl signal appeared at lower field in compound **3** (7.68 ppm) than in compound **5** (7.26 ppm); these differences can be attributed to the anisotropic effect of the carbonyl group. In compound **6**, the 6-H and 7-H signals appeared at lower field than the corresponding signals of compounds **5** and **3**, presumably due to the absence of the adjacent methyl group which exerts a shielding effect. Recently, the structure of synthetic **3** was established unambiguously by x-ray analysis (Nygjeral et al., 1975).

In the first step to synthesize model compounds of wyosine, compound **7** was prepared by condensation of guanosine and bromoacetone in the presence of sodium hydride. A 2′,3′-*O*-isopropylidene-5′-*O*-tosyl derivative of **7** was easily cyclized to **8** by heating in dioxane, which in turn was hydrolyzed by mild acid to **9**. Uv spectra of **9** are identical with those of synthetic **3**. This result verifies the 4,5′-cyclic structure of **8** and cleavage of the glycosidic bond in **8**. On the other hand, **7** was methylated with diazomethane to monomethyl derivative **10**. The site of methylation at N-5 in **10** was based on the following observations: (i) the

newly introduced methyl group is stable to acid treatment (possibility of O-methylation is excluded); (ii) the properties of the product are clearly different from those of quaternary ammonium salts; and (iii) clear differences are seen in the uv spectra between **10** and **8,** and between **11** and **9** or **3,** which exclude the methylation site being at N-4. Base **11** was obtained from **10** by vigorous acid hydrolysis. Attempted synthesis of wyosine by direct glycosidation of **3** gave, instead, the product that has identical uv spectra with **12** ob-



12, R = CH_3
13, R = ribofuranosyl

tained unequivocally by condensation of 3,7-dimethylguanine and bromoacetone. These results establish the *N*-1-ribosyl structure **13** for the glycosidation product.

Acknowledgment

We thank Dr. K. Hata of Jūjō Paper Co., Ltd., for the supply of *T. utilis* tRNA, and Professor H. G. Zachau and Dr. R. Thiebe for supplying wybutine and wybutoxine. We are also indebted to Dr. N. Wasada, Government Industrial Research Institute of Tokyo, for measurement of mass spectra, Mr. T. Kondo, Department of Agricultural Chemistry, Nagoya University, and Nippon Electric Varian Industry for measurement of NMR spectra.

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