# Structure of Wye (Yt Base) and Wyosine (Yt) from Torulopsis utilis Phenylalanine Transfer Ribonucleic Acid<sup>†</sup>

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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

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 tRNAPhe. 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<sup>1</sup> (wybutine, Y-Wye) could be liberated by mild acid treatment from brewer's yeast tRNA<sup>Phe</sup>, and that the Y-Wye-deficient tRNA<sup>Phe</sup> 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<sub>2</sub>Y-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 sidechain absolute configuration of Y-Wye has been determined as L (or S) and its synthesis has also been reported (Funamizu et al., 1971).

<sup>‡</sup> Present address: Institute of Microbial Chemistry, Kamiosaki 3-14, Shinagawa-ku, Tokyo, Japan. isolated from purified tRNA<sup>Phe</sup> 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.



From the studies on the nucleotide sequences in *Torulopsis utilis* tRNA<sup>Phe</sup> (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<sup>Phe</sup> (Takemura et al., 1974). This paper reports in detail the structural studies on wye and wyosine from *T. utilis* tRNA<sup>Phe</sup>.

## **Experimental Section**

*Materials.* BD-cellulose<sup>1</sup> 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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<sup>&</sup>lt;sup>1</sup> Abbreviations used are: tRNA<sup>Phe</sup>, phenylalanine specific tRNA; Y, the fluorescent nucleoside found in baker's yeast tRNA<sup>Phe</sup>; Yt, the fluorescent nucleoside found in *Torulopsis utilis* tRNA<sup>Phe</sup>; Yt, the fluorescent nucleoside found in *Torulopsis utilis* tRNA<sup>Phe</sup> (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;  $\Psi$ , pseudouridine; pA, 5'-adenylic acid; p $\Psi$ , 5'-pseudouridylic acid; BD-cellulose, benzoylated diethylaminoethyl-cellulose; DEAE-Sephadex, diethylaminoethyl-Sephadex; Ecteola-cellulose, ethylenechlorohydrintriethanolamine-cellulose; Me<sub>2</sub>SO, 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.

			Nucleoside			
Bravious	Ba	ise		Sym	bols	
Symbols	Name	Symbols	Name	3-Letter	1-Letter	
Yt. Yt <sup>+</sup>	Wye	Wye	Wyosine	Wyo	W	
Y, Y <sup>+</sup>	Wybutine	Y-Wye	Wybuto- sine	Y-Wyo	уW	
Peroxy Y, Yw, Yr	Wybutox- ine	O <sub>2</sub> Y-Wye	Wybuto- xosine	O <sub>2</sub> Y-Wyo	02yW	



FIGURE 1: Chromatography of *T. utilis* tRNA (10 g) on a column (5.2  $\times$  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<sub>2</sub> and 12 l. of 0.52 M NaCl-0.016 M MgCl<sub>2</sub> 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 (- - -).

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-propanolwater-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 HClwater (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  $A_{260}$ 



FIGURE 2: Chromatography of tRNA<sup>Phe</sup>-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<sub>2</sub> and 4 l. of 1 M NaCl-0.01 M MgCl<sub>2</sub>. 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<sup>Phe</sup> on a column (0.6  $\times$  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<sub>2</sub>-0.01 M Tris-HCl (pH 7.5) and 150 ml of 1 M NaCl-0.01 M MgCl<sub>2</sub>-0.01 M Tris-HCl (pH 7.5):  $A_{260}$  (—); phenylalanine acceptor activity (···); fluorescence intensity (-·-).

units) was charged with phenylalanine by crude phenylalanyl-tRNA synthetase from yeast and purified on a BD-cellulose column (2.2  $\times$  7.0 cm). Phenylalanyl-tRNA<sup>Phe</sup> 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<sup>Phe</sup> was deacylated and the free tRNA<sup>Phe</sup> (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<sub>1</sub> digest of highly purified tRNA<sup>Phe</sup> as described previously (Takemura et al., 1974). After removal of the 3'-terminal phosphate, the hexanucleotide  $(3.82 A_{260} \text{ units})$ was partially degraded with  $25 \mu g$  of snake venom phosphodiesterase at pH 8.4 and 37 °C for 6 h. The digest was chromatographed on a DEAE-cellulose column  $(0.25 \times 35 \text{ cm})$ 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 p $\Psi$ , was desalted, dephosphorvlated 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:  $\Psi$ , 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<sub>260</sub> unit of pure wyosine was obtained:  $\lambda_{max}$  (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-1H-imidazo[1,2-a]purine (3). 3-Methylguanine (500 mg) was suspended in 30 ml of 90% dimethylformamide (10% H<sub>2</sub>O). 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) ( $\epsilon$ ) 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<sub>2</sub>O) 3.69 ± 0.03 and  $8.46 \pm 0.02$ . Anal. Calcd for C<sub>9</sub>H<sub>9</sub>N<sub>5</sub>O: 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  $\alpha$ -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);  $\lambda_{max}^{pH 11.0}$  233 (35 000), 264 (5300), 307 (8000). pK<sub>a</sub> (in H<sub>2</sub>O)  $3.83 \pm 0.02$  and  $8.68 \pm 0.02$ . Anal. Calcd for C<sub>9</sub>H<sub>9</sub>N<sub>5</sub>O: 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<sub>2</sub>O) 3.30  $\pm$  0.02, 8.43  $\pm$  0.02. Anal. Calcd for C<sub>8</sub>H<sub>7</sub>N<sub>5</sub>O: C, 50.79; H, 3.73; N, 37.02. Found: C, 51.03; H, 3.57; N, 36.32.

3-(\beta-D-Ribofuranosyl)-5,9-dihydro-6-methyl-9-oxo-5Himidazo[1,2-a]purine (7). Guanosine (20.0 g) was dissolved in Me<sub>2</sub>SO (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<sub>2</sub>SO 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<sub>2</sub>SO and applied to a silica gel column (800 g). The product was eluted with a mixture of chloroform-methanol-Me<sub>2</sub>SO (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<sub>2</sub>SO-d<sub>6</sub>) δ 2.27 (6-CH<sub>3</sub>), 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 C13H15N5O5: C, 48.59; H, 4.71; N, 21.80. Found: C, 48.61; H, 4.81; N, 21.99.

3-(2',3'-O-Isopropylidene- $\beta$ -D-ribofuranosyl)-5,9-dihydro-6-methyl-9-oxo-5H-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 NaHCO3 (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<sub>2</sub>SO- $d_6$ )  $\delta$  1.31, 1.52 (isopropylidene CH<sub>3</sub>), 2.27 (6-CH<sub>3</sub>), 7.32 (7-H), 8.10 ppm (2-H). Anal. Calcd for C16H19N5O5: 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- $\beta$ -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.

	Solvent			
Compound	A	В	С	
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 icewater (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<sub>3</sub>)  $\delta$  1.35, 1.55 (isopropylidene CH<sub>3</sub>), 2.26 (6-CH<sub>3</sub>), 7.20 (7-H), and 7.55 (2-H) ppm; uv  $\lambda_{max}^{\text{pH 0.91}}$ 231 (36 500), 277 (13 800);  $\lambda_{max}^{\text{pH 6.50}}$  236 (34 300), 295 (8900). Anal. Calcd for C<sub>16</sub>H<sub>17</sub>N<sub>5</sub>O: 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-( $\beta$ -D-Ribofuranosyl)-5,9-dihydro-5,6-dimethyl-9-oxoimidazo[1,2-*a*]purine (10). Compound 7 (200 mg) was dissolved in Me<sub>2</sub>SO-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<sub>2</sub>SO-d<sub>6</sub>)  $\delta$  2.31 (6-CH<sub>3</sub>), 3.57 (5-CH<sub>3</sub>), 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<sub>14</sub>H<sub>17</sub>N<sub>5</sub>O<sub>5</sub>: 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)  $\delta$  2.05 (6-CH<sub>3</sub>), 3.42 (5-CH<sub>3</sub>), 7.10 (7-H), and 8.23 ppm (2-H); uv  $\lambda_{max}^{\text{pH 6.5}}$  230, 293;  $\lambda_{max}^{\text{pH 2.0}}$  223, 235 (sh), 273, 302.5;  $\lambda_{max}^{\text{pH 10.9}}$  234, 267 (sh), 300. Anal. Calcd for C<sub>9</sub>H<sub>9</sub>N<sub>5</sub>O: 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<sub>2</sub>SO (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 mix-



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

ture 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<sub>3</sub>)  $\delta$  2.33 (6-CH<sub>3</sub>), 3.90 (N-CH<sub>3</sub>), 4.18 (N-CH<sub>3</sub>), 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<sub>10</sub>H<sub>11</sub>N<sub>5</sub>O: C, 55.29; H, 5.10; N, 32.24. Found: C, 55.12; H, 5.08; N, 32.38.

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- $\beta$ -D-ribofuranose (8.0 g) was added ptoluenesulfonic 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<sub>2</sub>O) 3.40 ± 0.02. Anal. Calcd for C14H17N5O5: 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<sub>3</sub>), 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  $(\cdots)$ ; 0.1 N NaOH  $(\cdots)$ .

203.0802 (M calculated for C<sub>9</sub>H<sub>9</sub>N<sub>5</sub>O: 203.0807), 202.0730 (C<sub>9</sub>H<sub>8</sub>N<sub>5</sub>O: 202.0729), 188.0590 (C<sub>8</sub>H<sub>6</sub>N<sub>5</sub>O: 188.0572), and 174.0767 (C<sub>8</sub>H<sub>8</sub>N<sub>5</sub>: 174.0780). The spectrum suggests that wye has the molecular formula C<sub>9</sub>H<sub>9</sub>N<sub>5</sub>O 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.  $3.66 \pm 0.05$  and  $8.52 \pm 0.10$ , 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  $\delta$  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<sub>3</sub>-C=C-H grouping. The above data

Table III:	$R_f$ Values	of Avicel	SF Cellulose	Thin-Layer
Chromato	ography.			

	Solvent					
Compd	В	G	Н	F	С	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
Compoun	$d 3 in D_2$	). <i>a</i>					

	6-CH <sub>3</sub>	4-CH <sub>3</sub>	7-H	2-Н
Wye	2.24	3.82	7.32	8.07
3	2.26	3.82	7.30	8.09

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<sub>6</sub> and C<sub>7</sub>, respectively, and the N-methyl group at N<sub>4</sub> (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 3methylguanine and  $\alpha$ -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<sub>2</sub>O. 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 iden-



tical 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-



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

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