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Covalent Attachment of Fluorescent Groups to Transfer Ribonucleic Acid. Reactions with 4-Bromomethyl-7-methoxy-2-oxo-2*H*-benzopyran†

Chih-hsin Yang‡ and Dieter Söll*

ABSTRACT: Pseudouridine in *Escherichia coli* $tRNA^{fMet}$ and 2-thio-5-(*N*-methylaminomet hyl)uridine in *E. coli* $tRNA^{Glu}$ were specifically modified with the fluorescent compound 4-bromomethyl-7-methoxy-2-oxo-2*H*-benzopyran. In reactions with the homologous aminoacyl-tRNA synthetases the modified tRNAs showed acceptor activities of 30 and 10%, respectively, compared to unmodified tRNA.

The chemical modification of tRNA presents a great challenge to organic chemists. Specificity for a particular base and reaction at only one site in the molecule are required if a useful derivative is to be obtained. Such procedures are important in the purification of tRNA species (Gillam *et al.,* 1968). Modified tRNA molecules have aided structural studies on tRNA by fluorescence spectroscopy (Cantor and Tao, 1971), X-ray crystallography (Kim *et al.,* 1973), or electron spin resonance (Kabat *et al.,* 1970; Hara *et al.,* 1970). In addition, these compounds are invaluable in investigating the relationship of structure to biological function of tRNA (see, *e.g.,* Thiebe and Zachau, 1968). The majority of the chemical modification reactions involve the free amino group of the amino acid in aminoacyl-tRNA or the modified nucleosides in tRNA. Not only is their reactivity often higher than those of the four major mononucleotides, but since they only occur once or twice in a tRNA molecule a site specific reaction is more easily obtained.

Continuing our studies on the chemical modification of tRNA with fluorescent groups, we have further investigated the use of 4-bromomethyl-7-methoxy-2-oxo-2*H*-benzopyran (BMB).[1] This compound was described by Secrist *et. al.* (1971) as a specific reagent for 4-thiouridine. We have successfully modified 4-thiouridine in tRNA with this fluorescent group (Yang and Söll, 1973b). In this communication we show that BMB reacts readily also with pseudouridine and 2-thio-5-(*N*-methylaminomet hyl)uridine. Thus, we were able to modify the latter nucleoside in *Escherichia coli* $tRNA^{Glu}$ and the single pseudouridine residue in *E. coli* $tRNA^{fMet}$.

Materials and Methods

General. Uniformly labeled [^{14}C]methionine and [^{14}C]glutamate with a specific activity of 221 and 197 Ci/mol, respectively, were obtained commercially. Pseudouridine and pseudouridine 3′-phosphate were purchased from Sigma. 4-Bro-

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[1] Abbreviations used are: BMB, 4-bromomethyl-7-methoxy-2-oxo-2*H*-benzopyran; BD-cellulose, benzoylated DEAE-cellulose; Nucleotides and nucleosides are abbreviated according to the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (*Biochemistry 9,* 4022 (1970)). ! denotes 2′,3′-cyclic phosphate; ^{2}Sp*, 2-thio-5-(*N*-methylaminomet hyl)uridine 3′-phosphate. One optical density unit at 260 nm (A_{260} unit) is the amount of material/ml of a solution which produces an absorbance of 1 in a 1-cm light path at 260 nm. ^{2}S*-BMB-tRNA designates tRNA to which a BMB residue is attached covalently through the ^{2}S*. ψ-BMB-tRNA designates tRNA to which a BMB residue is attached covalently through the ψ.

TABLE I: R_F Values and Electrophoretic Mobilities.

Compounds	Chromatographic Solvent Systems A	B	C	Electrophoresis R_m (Relative to pU)
		R_F Values		
1. BMB	0.82	0.99	0.87	0.12
2. Adduct of BMB and ^{2}Sp* (T_2 digest of ^{2}S*-BMB-tRNA^{Glu})	0.56	0.81		
3. Adduct of BMB and ^{2}Sp* (T_2 digest of $\psi,\psi,^2$S*-BMB-tRNA^{Glu})	0.59	0.69		
4. Adduct of BMB and pseudouridine 3′-phosphate	0.57	0.97		
5. Pseudouridine 3′-phosphate	0.28	0.53		0.97
6. Adduct of BMB and pseudouridine	0.70	0.72		
7. Pseudouridine	0.43	0.36		0.25
8. Adduct of BMB and 2-thiouracil	0.90	0.86		0.19
9. 2-Thiouracil	0.59	0.63	0.62	0.10
10. Adduct of BMB and 5-(*N*-methylaminomethyl)uracil	0.78	0.38	0.72	0.12
11. 5-(*N*-Methylaminomethyl)uracil	0.69	0.24	0.26	−0.18[a]

[a] Moves toward the cathode.

momethyl-7-methoxy- 2-oxo-2*H*-benzopyran was kindly given by Dr. N. J. Leonard. 5-Methylaminomethyluracil was a gift of Dr. J. Carbon. BD-cellulose was a product of Boehringer-Mannheim. Purified *E. coli* (MRE600) tRNA^{fMet} was a product of Boehringer-Mannheim (kindly given to us by Dr. G. Weimann) and had a methionine acceptor activity of 1.7 nmol/A_{260} unit). Purified *E. coli* tRNA$^{Glu}_2$ with an acceptor activity of 1.2 nmol/A_{260} unit and *E. coli* tRNA^{fMet} (specific activity 1.5 nmol/A_{260} unit) was kindly provided by Dr. A. D. Kelmers from the Oak Ridge National Laboratory. Pure methionyl-tRNA synthetase (mol wt of monomer 90,000) and glutamyl-tRNA synthetase (mol wt 56,000) from *E. coli* K12 (strain CA244) were prepared as described in the literature (Lemoine *et al.*, 1968; Lapointe and Söll, 1972). Both enzyme preparations were approximately 95% pure as judged by native and sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Chromatography. Thin-layer chromatography (tlc) and electrophoresis were routinely used for characterization of the nucleosides or nucleotides obtained by T_2 RNase digests of tRNA or RNase T_1 oligonucleotides. Ascending tlc (cellulose) was run in the following systems: (A) isobutyric acid–concentrated NH_4OH–H_2O, 66:1:33; (B) isopropyl alcohol–concentrated HCl–H_2O, 70:15:15; (C) 1-butanol–ethanol–H_2O, 50:17:35. Thin-layer electrophoresis (on cellulose) was run in 20 mM potassium phosphate (pH 7.0). For preparative purposes descending paper chromatography on Whatman 3MM paper was used.

Assay for Amino Acid Acceptor Activity. The incubation mixture contained per milliliter: 100 μmol of sodium cacodylate (pH 7.2), 10 μmol of magnesium acetate, 10 μmol of potassium chloride, 2 μmol of ATP, 0.05 A_{260} unit of tRNA, 4 nmol of radioactive amino acid, and aminoacyl-tRNA synthetase preparation. After incubation at 37°, aliquots were removed and the acid-insoluble radioactivity was determined by the filter paper technique.

Enzymatic Digestion of tRNA. These were performed with ribonucleases A, T_1, T_2, bacterial alkaline phosphatase, or snake venom phosphodiesterase as described by Barrell (1971).

DEAE-Cellulose Column Chromatography in the Presence of Urea. Oligonucleotides from complete RNase T_1 digests of tRNA were separated as described by Tener (1967). The column was developed at pH 7.5. The oligonucleotides were freed from urea by adsorbing them to DEAE-cellulose after fivefold dilution with water and eluting them with 1.0 M triethylammonium bicarbonate (pH 8). The recovered oligonucleotides were analyzed by thin-layer chromatography after complete ribonuclease T_2 digestion.

Absorption and Steady-State Fluorescence Measurements. Absorption spectra were measured on a Cary Model 15 recording spectrophotometer using a 10-mm light-path cuvet. The absorption spectrum of the fluorescent group on tRNA was measured against a solution of identical buffer and tRNA concentration in the reference compartment. Steady-state fluorescence spectra were obtained on the fluorospectrophotometer designed in the laboratory of Dr. L. Stryer using 10 × 10 mm cuvets. The absorbance of the sample at excitation wavelength was usually below 0.05 in order to minimize the inner filter effect. All measurements were made at 24°. The excitation spectra were corrected directly by using a ratio amplifier. The emission spectra were not corrected for the variation in the sensitivity of the detection system with the wavelength except for the quantum yield measurements. Absolute quantum yields were obtained by the method of Weber and Teale (1957). Quinine sulfate in 1 N sulfuric acid (quantum yield of 0.58) was used as a standard (Turro, 1967).

Nanosecond Fluorospectroscopy. The nanosecond kinetic measurements were performed on a nanosecond spectrofluorometer built in the laboratory of Dr. L. Stryer. The mechanism and the design of the spectrofluorometer were described in detail by Yguerabide *et al.* (1970). Corning filters 7-60 and 3-73 were used for selecting excitation and emission light respectively for BMB and its derivatives. The lifetimes of fluorophores were determined as described by Yguerabide (1972).

Reaction of Pseudouridine or Pseudouridine 3′-Phosphate with BMB. Pseudouridine (10 mg) and 10 mg of BMB were dissolved in 5 ml of 75% dimethyl sulfate in 0.1 M potassium phosphate (pH 8.4) and incubated at 60° for 5 hr. The reaction only proceeded up to 50%. The adduct of pseudouridine and BMB was purified by paper chromatography on 3MM paper in solvent system B. The reaction between pseudouridine 3′-phosphate and BMB was performed under identical conditions. The R_F values of the adducts of BMB with pseudouridine or pseudouridine 3′-phosphate are shown in Table I.

Reaction of 2-Thiouracil or 5-Methylaminomethyluracil with BMB. These were performed under conditions identical with those observed for ψp-BMB. The R_F values of the products in different solvent systems are shown in Table I.

Conversion of 4-Thiouridine in Uridine in tRNA. Quantita-

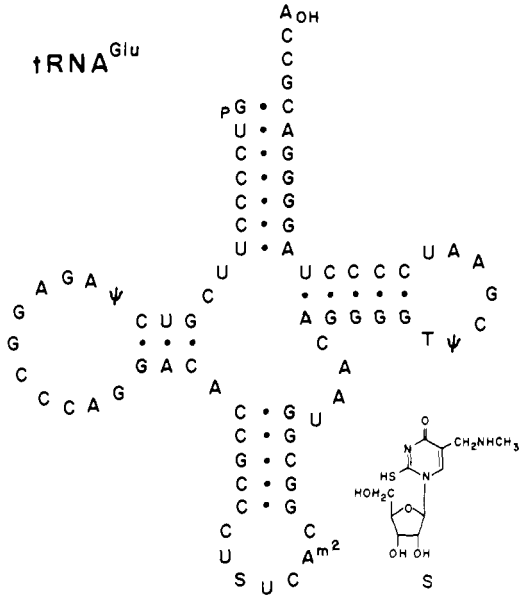

FIGURE 1: Structure of *E. coli* $tRNA^{Glu}_2$ (Ohashi *et al.*, 1972). S = 2-thio-5-(*N*-methylaminomet hyl)uridine.

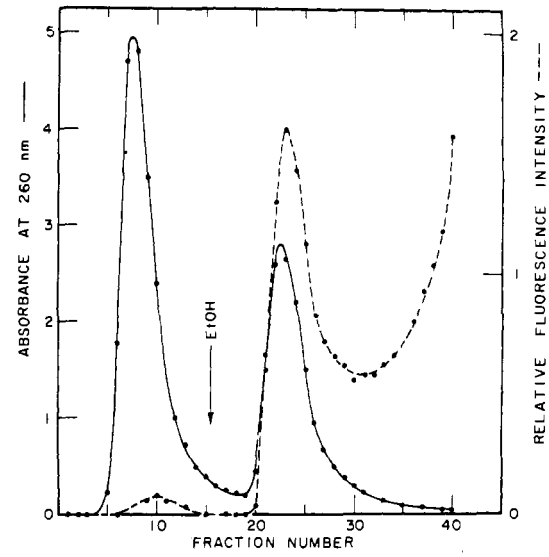

FIGURE 2: Purification of $^2S^*$-BMB-$tRNA^{Glu}$. The tRNA recovered after reaction (about 60 A_{260} units) was applied to a BD-cellulose column (0.6 × 20 cm) which was previously equilibrated with 0.4 M NaCl–10 mM sodium acetate (pH 4.5)–10 mM $MgCl_2$. The column was developed as described in Materials and Methods. Fractions of 3 ml were collected every 5 min.

tive conversion of 4-thiouridine in $tRNA^{fMet}$ to uridine with cyanogen bromide was performed in exactly the same way as described by Walker and RajBhandary (1972).

Covalent Attachment of BMB to tRNA. In aqueous solution: 60 A_{260} units of $tRNA^{Glu}$ in 1 ml of 0.1 M potassium phosphate (pH 8.4) was incubated with 12.5 mg of BMB adsorbed on Celite (3:10) under constant shaking for 24 hr at 37°. The excess BMB and Celite was removed by filtration through a nitrocellulose filter (Millipore) and successive alcohol precipitations. The modified tRNA was purified by BD-cellulose chromatography as described below. Under these conditions, incomplete reaction (~40%) of 2-thio-5-(*N*-methylaminomethyl)uridine in $tRNA^{Glu}$ occurred while there was no reaction of ψ with BMB.

Under denaturing conditions, the incubation mixture (0.4 ml) contained per ml: 50 A_{260} units of tRNA, 25 μmol of potassium phosphate (pH 6.5), and 3.8 mg of BMB in 75% dimethyl sulfoxide. The mixture was kept at 37° for 5 hr. Then water was added to decrease the concentration of dimethyl sulfoxide and the reaction mixture was dialyzed extensively against 10 mM $MgCl_2$–10 mM sodium phosphate (pH 7.0). The modified tRNA was purified by BD-cellulose chromatography as described below. Under these conditions, pseudouridine and 2-thio-5-(*N*-methylaminomethyl)uridine in $tRNA^{Glu}$ reacted with BMB.

Purification of the Modified tRNA. The tRNA recovered after reaction (about 10–60 A_{260} units) was applied to a BDC column (0.6 × 20 cm) which was previously equilibrated with 0.4 M NaCl–10 mM sodium acetate (pH 4.5)–10 mM $MgCl_2$. The column was then eluted with gradient of 0.4–1.5 M NaCl in a buffer of 10 mM $MgCl_2$–10 mM sodium acetate (pH 4.5); then a gradient of 0–40% alcohol in 1.5 M NaCl–10 mM $MgCl_2$–10 mM sodium acetate (pH 4.5); and finally 40% alcohol in 1.5 M NaCl–10 mM $MgCl_2$–10 mM sodium acetate (pH 4.5).

Results

1. *Attachment of BMB to2-Thio-5-(N-methylaminomet hyl)uridine in $tRNA^{Glu}$ in Aqueous Solution.* (a) PREPARATION OF $^2S^*$-BMB-$tRNA^{Glu}$. *E. coli* $tRNA^{Glu}$ contains 2-thio-5-(*N*-methylaminomet hyl)uridine in the anticodon (Figure 1). This nucleoside is reactive toward cyanogen bromide and acylating reagents (Agris *et al.*, 1973; Cedergren *et al.*, 1973). When $tRNA^{Glu}$ was dissolved in 0.1 M potassium phosphate (pH 8.5) and incubated with BMB adsorbed on Celite (Secrist *et al.*, 1971), BMB became covalently linked to the tRNA. After removal of the free dye, the product was analyzed by BD-cellulose chromatography (Figure 2). The unreacted $tRNA^{Glu}$ eluted in the salt gradient and did not contain significant amounts of fluorescence. The tRNA which had reacted with BMB was eluted from the BD-cellulose only when 10% ethanol was present in the eluent. The profile of fluorescence intensity coincided with that of ultraviolet absorbance indicating a good separation of modified $tRNA^{Glu}$ from unreacted $tRNA^{Glu}$. The yield was about 40%.

(b) CHEMICAL CHARACTERIZATION OF $^2S^*$-BMB-$tRNA^{Glu}$. When the $^2S^*$-BMB-$tRNA^{Glu}$ was subjected to tlc in solvent B the fluorescent spot remained at the origin indicating that BMB was indeed covalently linked to the tRNA. When a complete T_2 ribonuclease digest of $^2S^*$-BMB-$tRNA^{Glu}$ was analyzed by tlc in solvent B only one fluorescent spot was found indicating that the reaction was specific for one particular base. This was confirmed by the analysis of the complete T_1 ribonuclease digest of $^2S^*$-BMB-$tRNA^{Glu}$ by DEAE-cellulose chromatography (Figure 3). The material in each peak was analyzed by T_2 ribonuclease digestion, followed by tlc in solvents A and B. The base analysis results showed that our elution pattern was identical with that of T_1 hydrolysis products of this tRNA as reported by Munninger and Chang (1972). The fluorescence intensity corresponded to the decanucleotide which contained 2-thio-5-(*N*-methylaminomethyl)uridine. The smaller fluorescence peak represents the 2′,3′-cyclic phosphate ended decanucleotide as shown by base analysis. Since no major mononucleotide reacted with BMB we concluded that the BMB was covalently linked to 2-thio-5-(*N*-methylaminomethyl)uridine in $tRNA^{Glu}$.

2. Attachment of BMB to $tRNA^{Glu}$ under Denaturing Conditions. a. PREPARATION OF ψ,ψ, $^2S^*$-BMB-$tRNA^{Glu}$. Since the reaction of $tRNA^{Glu}$ with BMB in aqueous solution gave only 40% yield the reaction was repeated under denaturing

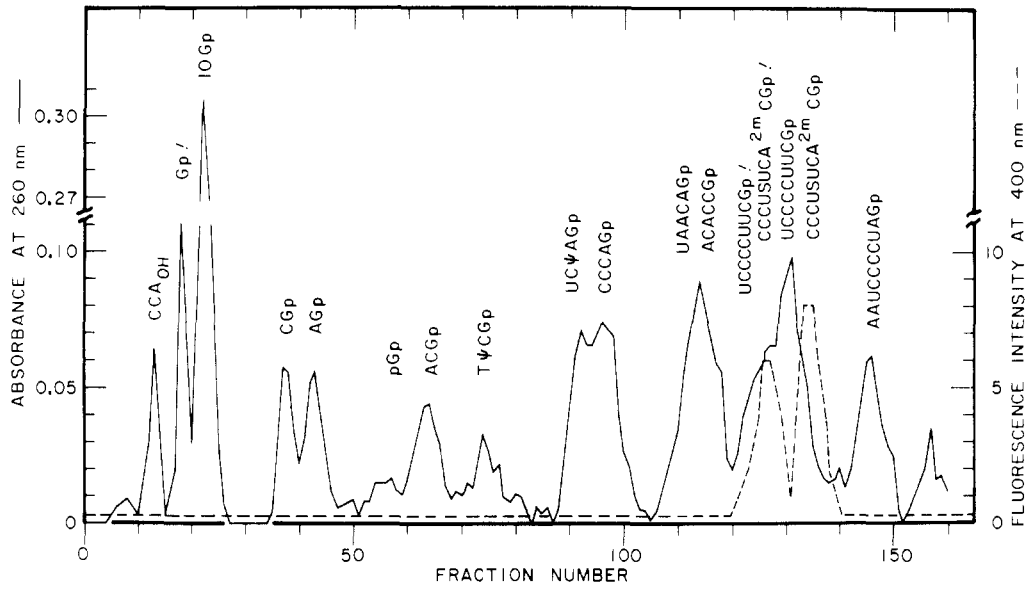

FIGURE 3: DEAE-cellulose column chromatography of a complete ribonuclease T_1 digest of ^{2}S*-BMB-tRNA^{Glu}. The digest of 10 A_{260} units of tRNA was applied to a DEAE-cellulose column (0.3 × 80 cm) which was preequilibrated with 20 mM Tris-HCl (pH 7.5)–7 M urea. The column was then eluted with a linear gradient of 0–0.45 M NaCl in 20 mM Tris-HCl (pH 7.5)–7 urea. Total volume of the gradient was 500 ml. Fractions of 1.5 ml were collected every 15 min.

conditions using 75% aqueous dimethyl sulfoxide and BMB in homogeneous solution. Analysis of the reaction mixture by BD-cellulose chromatography showed the reacted tRNA^{Glu} could be eluted from the column only in the presence of 30% alcohol in the elution buffer, while ^{2}S*-BMB-tRNA^{Glu} had a weaker affinity for BD-cellulose and eluted at lower alcohol concentrations. Unreacted *E. coli* tRNA^{Glu} was eluted in the early salt fractions from this column. The fluorescence peak coincides with the absorbance peak in the alcohol fractions indicating good separation of BMB-tRNA^{Glu} from unreacted tRNA^{Glu}. Comparison of the BD-cellulose chromatography profile of ψ,ψ,^{2}S*=BMB-tRNA^{Glu} with that of ^{2}S*-BMB-tRNA^{Glu} suggested that more than one BMB residue was covalently linked to tRNA^{Glu} in ψ,ψ,^{2}S*-BMB-tRNA^{Glu}. The absorbance at 325 nm of this tRNA and the ratio of the fluorescence intensity (at 400 nm) to absorbance at 260 nm indicated that there were three BMB molecules per tRNA^{Glu} molecule (relative to ψ-BMB-tRNA^{fMet}).

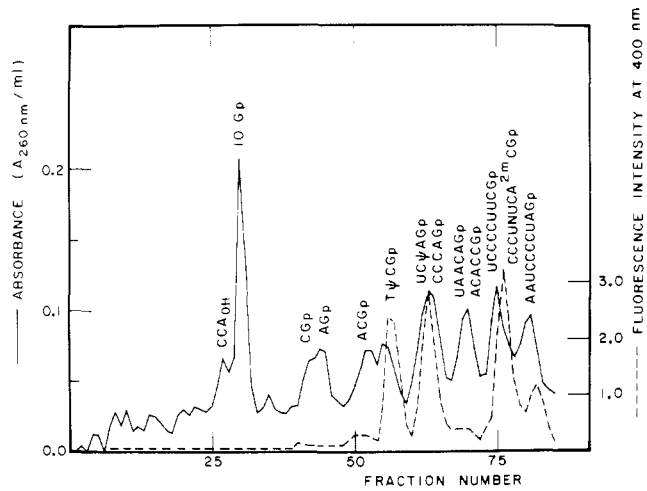

FIGURE 4: DEAE-cellulose column chromatography of a complete ribonuclease T_1 digest of ψ,ψ,^{2}S*-BMB-tRNA^{Glu}. The digest of 10 A_{260} units was applied and the column was developed as described in the legend to Figure 3. Fractions of 3 ml were collected every 30 min.

b. CHEMICAL CHARACTERIZATION OF ψ,ψ,^{2}S*-BMB-tRNA^{Glu}. When this tRNA was subjected to tlc in solvent B, the fluorescent spot remained at the origin indicating that BMB was covalently linked to the tRNA. The complete T_2 ribonuclease digest of ψ,ψ,^{2}S*-BMB-tRNA^{Glu} showed two fluorescent spots when analyzed by two-dimensional tlc. In order to determine the exact positions of the nucleotides which had reacted with BMB, a complete ribonuclease T_1 digest of ψ,ψ,^{2}S*-BMB-tRNA^{Glu} was analyzed by DEAE-cellulose chromatography. The elution pattern (Figure 4) showed three fluorescence peaks of about equal intensity. The material in each fluorescent peak was digested with T_2 ribonuclease and analyzed by two-dimensional tlc. The oligonucleotides T-ψ-C-Gp, U-C-ψ-A-Gp, and C-C-C-U-S-U-C-m^{2}A-C-Gp contained fluorescence. The material in the additional small fluorescent peak was not examined. The analysis showed further that pseudouridine and 2-thio-5-(*N*-methylaminomethyl)uridine had reacted with BMB in ψ,ψ,^{2}S*-BMB-tRNA^{Glu}.

3. The Adduct of Pseudouridine and BMB and Its Structure. In order to elucidate unambiguously the structure of the reaction product of BMB and pseudouridine and also to have an authentic marker compound for comparison, reactions of pseudouridine 3′-phosphate or pseudouridine with BMB were carried in aqueous dimethyl sulfoxide as described in Materials and Methods. It should be noted that much more drastic reaction conditions (high temperature) were needed to form the addition products of these compounds compared to the ease of reaction with pseudouridine contained in tRNA. The R_F values of the synthetic product on tlc or thin-layer electrophoresis are shown in Table I. The adduct of pseudouridine 3′-phosphate and BMB is identical with the product isolated during the base analysis of the oligonucleotides T-ψ-C-Gp and U-C-ψ-A-Gp. The chemically synthesized adduct of pseudouridine and BMB was further characterized unambiguously to show the linkage between the fluorescent dye and pseudouridine. By comparison of the difference spectra (see Figure 5) of ψ-BMB and BMB in acid and alkali it is clear that the ionizable proton at N-1 is lost since the spectrum resembles that of uridine. Thus, BMB is linked through the N-1 position of pseudouridine and the structure of the adduct is as shown in Figure 6.

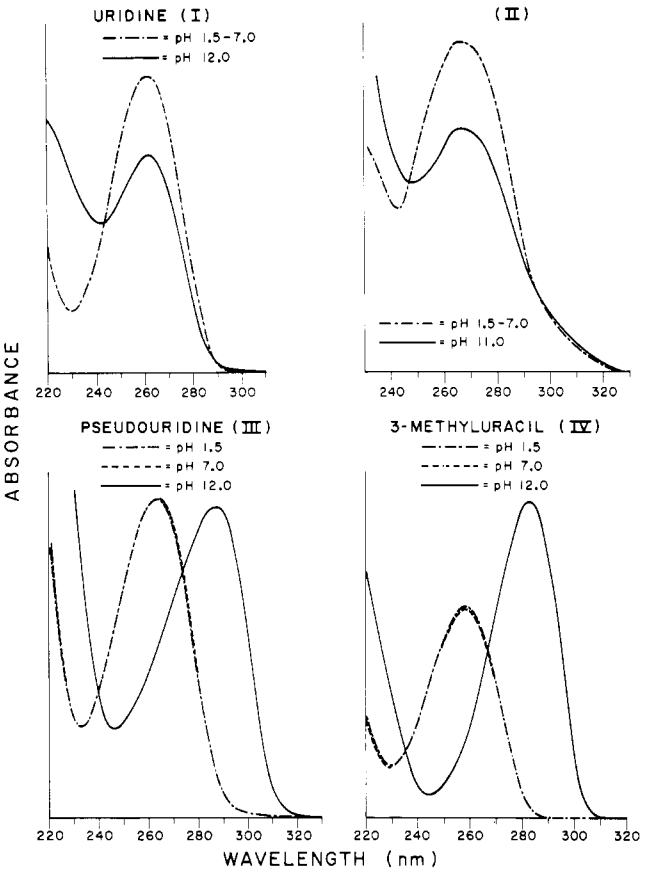

FIGURE 5: Difference uv spectrum (II) of ψ-BMB and free BMB compared to the uv spectra of uridine (I), 3-methyluracil (III), and pseudouridine (IV). This is a spectrum of the adduct of pseudouridine and BMB from which the spectrum of an equivalent amount of BMB (as estimated by the fluorescence intensity at 400 nm) has been subtracted. The spectra of the other compounds are taken from Hall (1971).

4. Attachment of BMB to ψ in E. coli tRNA$^{fMet}_U$. a. PREPARATION OF ψ-BMB-tRNA$^{fMet}_U$. The easy reaction of BMB with pseudouridine suggested a way to introduce a fluorescent dye into the T-ψ-C loop of tRNA. We choose *E. coli* tRNA^{fMet} since it contains only one pseudouridine (Dube and Marcker, 1969). However, under the reaction conditions required BMB would react both with the 4-thiouridine (Yang and Söll, 1973b) and the pseudouridine residue in this tRNA. Therefore the 4-thiouridine in tRNA was converted to uridine by desulfurization with cyanogen bromide to form tRNA$^{fMet}_U$ (Walker and RajBhandary, 1972). This reaction is specific to 4-thiouridine and does not affect the biological activity of tRNA^{fMet}. tRNA$^{fMet}_U$ was coupled with BMB in 75% dimethyl sulfoxide under the same conditions as the preparation of ψ,ψ,^{2}S*-BMB-tRNA^{Glu}. After removing free BMB, ψ-BMB-tRNA$^{fMet}_U$ was purified by BD-cellulose chromatography.

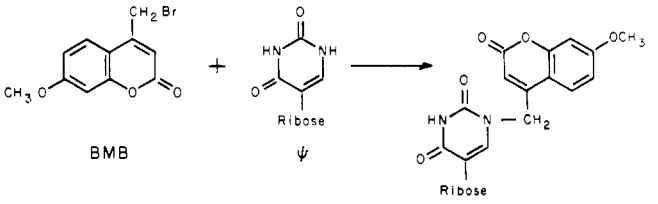

FIGURE 6: Reaction scheme of BMB and pseudouridine.

b. CHEMICAL CHARACTERIZATION OF ψ-BMB-tRNA$^{fMet}_U$. ψ-BMB-tRNA$^{fMet}_U$ was digested with ribonuclease T_2 and the hydrolysate analyzed by two-dimensional tlc in solvents A and B. The authentic ψ-BMB (see above) was treated similarly as a marker. The only fluorescent spot on the tlc plate detectable had the same R_F values as the marker compound. This confirmed that BMB was indeed attached to ψ in ψ-BMB-tRNA$^{fMet}_U$.

5. Properties of BMB-tRNAs. a. AMINOACYLATION OF MODIFIED tRNAs. For any further work with these modified tRNAs it is crucial to know whether they still possess biological activity after the various chemical treatments. Therefore we examined their capacity to be aminoacylated by preparations of pure homologous aminoacyl-tRNA synthetases (Table II).

Native tRNA^{Glu} preparations had a specific activity of 1.2 nmol/A_{260} unit (about 70% pure). Single modification with BMB in the anticodon of tRNA^{Glu} reduced the extent of enzymatic aminoacylation to 10% of the level obtained with unmodified tRNA. This is not surprising since as suggested by Agris *et al.* (1973) ^{2}S* is involved in the interaction between tRNA^{Glu} and its cognate aminoacyl-tRNA synthetase. The addition of two more BMB residues prevents the aminoacylation of ψ,ψ,^{2}S*-BMB-tRNA^{Glu}. We do not believe that the 10% remaining acceptor activity of ^{2}S*-BMB-tRNA^{Glu} is due to a contaminant of unmodified tRNA in our preparation since BD-cellulose chromatography should have effected a separation. A 10% contamination in our preparation by a differently

TABLE II: Properties of Modified tRNAs.[a]

	No. of Fluorescent Groups per tRNA	Extent of Amino-acylation (nmol/A_{260} unit)	$\epsilon_{max} \times 10^{-3}$	λ_{max}^{ex} (nm)	λ_{max}^{em} (nm)	Lifetime τ (nsec)	Rotational Correlation Time ρ (nsec)
tRNA^{fMet} (unmodified)	0	1.5					
ψ-BMB-tRNA$^{fMet}_U$	1	0.5	12.5 (315)[b]	326	400	1.8	8.5
tRNA^{Glu} (unmodified)	0	1.2					
^{2}S*-BMB-tRNA^{Glu}	1	0.12	15.4 (325)	324	400	1.8	
ψ,ψ,^{2}S*-BMB-tRNA^{Glu}	3	0	37 (325)	325	400	1.8	8.0

[a] Optical measurements were performed in 10 mM potassium phosphate (pH 7.0)–10 mM $MgCl_2$. tRNA concentrations were between 0.2 and $\sim 2 \times 10^{-6}$ M. [b] The numbers in parentheses show the wavelength at which extinction coefficients were determined.

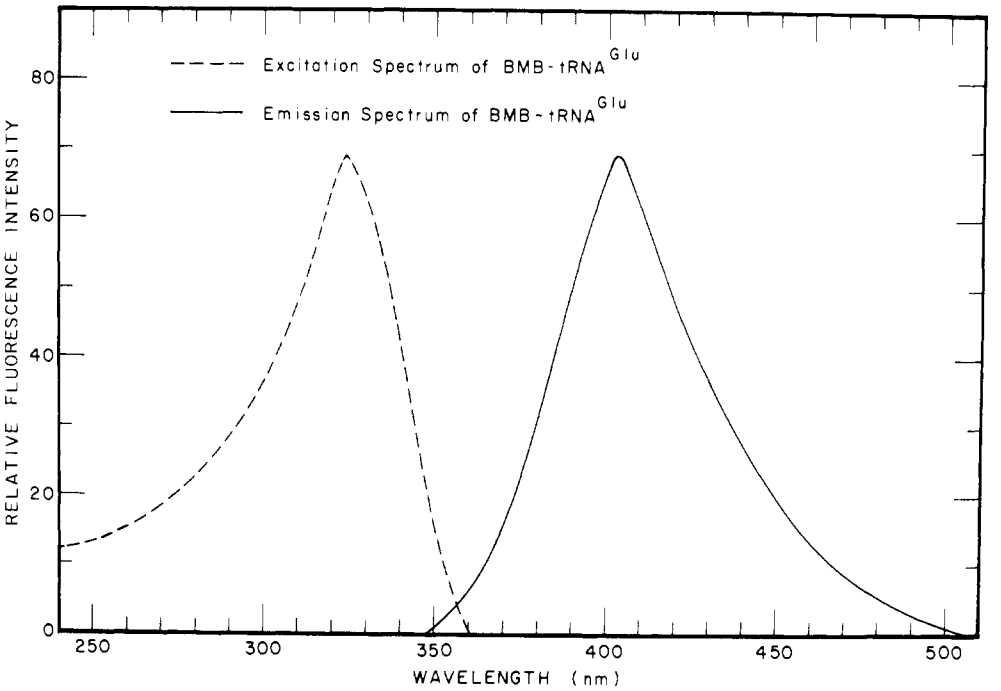

FIGURE 7: Optical properties of $\psi,\psi,^2$S*-BMB-tRNA^{Glu}. The excitation spectrum measured at 400 nm (- -); the emission spectrum excited at 320 nm (—). The tRNA was dissolved in 10 mM potassium phosphate (pH 7.0)–10 mM $MgCl_2$.

modified, fully chargeable tRNA is unlikely but difficult to rule out on the basis of oligonucleotide analysis.

Native tRNA^{fMet} preparations had a specific activity of 1.5–1.7 nmol/A_{260} unit indicating at least 90% purity. As shown in Table II, ψ-BMB-tRNA$^{fMet}_U$ could be aminoacylated to 30% of the level obtained using native tRNA^{fMet}. Since it is known that tRNA^{fMet} retains full ability to be charged after conversion of 4-thiouridine to uridine (Walker and RajBhandary, 1972) this effect must be ascribed to the reaction of pseudouridine with BMB. It has been reported that modification of the same nucleoside by acrylonitrile completely destroys the amino acid acceptor activity of this tRNA (Siddiqui *et al.*, 1970). The reason for the discrepancy in our results is unclear at present.

The incomplete aminoacylation observed in these studies may be a general phenomenon, due to the altered rates of aminoacylation and enzymatic deacylation (Bonnet and Ebel, 1972) when chemically modified tRNAs are used (Yang and Söll, 1973a).

b. Spectral Properties of Modified tRNAs. The absorption spectra of the fluorescent moiety in the modified tRNA were determined against a solution of identical buffer and tRNA concentration in the reference compartment. The extinction coefficients and the wavelengths used for measurements of the modified tRNAs are shown in Table II. The extinction coefficients of the dyes in the modified tRNAs were determined experimentally assuming that the fluorescent moiety and the tRNA were present in stoichiometric amounts and an absorbance (A_{260}) of 0.6 (in water) for 1 nmol of tRNA. The measured values agree well with that of the free dye compound. The excitation and the emission spectra of the BMB-tRNAs were comparable with those of the free dye. The excitation spectra of BMB-tRNA^{Glu} were slightly different from the former, having a higher leading edge of the peak (Figure 7). The rotational correlation times of the BMB moiety in the tRNAs (Table II) are short compared to that of the whole tRNA molecule which is 25 nsec (Cantor and Tao, 1971).

Discussion

We have prepared three species of tRNA with covalently attached fluorescent groups: ^{2}S*-BMB-tRNA^{Glu}, ψ-BMB-tRNA$^{fMet}_U$, and $\psi,\psi,^2$S*-BMB-tRNA^{Glu}. The modified tRNAs were obtained in pure form after separation from the unreacted tRNA on BD-cellulose chromatography, and the position of the modified nucleotide in the tRNA sequence was determined. During these studies the following interesting points arose.

BMB was described as a specific reagent for 4-thiouridine by Secrist *et al.* (1971), but in addition to its reaction with 2-thiouridine derivatives we found that it also reacts with pseudouridine. The reactivity toward thionucleosides is higher than toward pseudouridine since it is possible to obtain ^{4}S-BMB-tRNA^{fMet}, in which the pseudouridine is unmodified (Yang and Söll, 1973b). In aqueous solution the BMB reaction appears to be specific for nucleosides in "exposed" regions, modifying only the ^{2}Sp* in the anticodon loop of tRNA^{Glu} but not the two pseudouridines found elsewhere in the tRNA. However, if the tRNA is denatured in organic solvents, all three nucleosides are reactive.

Since samples of 2-thio-5-(*N*-methylaminomethyl)uridine or any nucleotide derivative were not available to us, it was not possible to characterize the exact structure of its adduct with BMB. Two possible reaction products might be expected. BMB could be linked through a thioether bridge to C_2 of uridine, or alternatively could react to give the alkylation product of the secondary amino nitrogen of the 5-methylaminomethyl group. We have shown that 5-methylaminomethyluracil reacts with BMB, and it has been shown that the exocyclic amino group can be acylated (Cedergren *et al.*, 1973). Two chromatographically different ^{2}Sp*-BMB adducts were formed when the reaction of BMB with tRNA^{Glu} was performed under denaturing conditions or in aqueous solution (Table I). Variations in fluorescence intensity ($\psi,\psi,^2$S*-BMB-tRNA^{Glu} with three BMB residues per tRNA molecule shows ten times greater fluorescence than ^{2}S*-BMB-tRNA^{Glu}) and different stabilities

toward acid (the adduct isolated from $\psi,\psi,^2$S*-BMB-tRNA^{Glu} is more stable in HCl than in adduct ^{2}S*-BMB-tRNA^{Glu}) lend support to the idea that two different structural adducts are formed under aqueous and denaturing reaction conditions.

Another interesting observation was the fact that the reaction of BMB with isolated pseudouridine 3′-phosphate required higher temperature and longer reaction times than its reaction with pseudouridine in intact tRNA molecules. This resembles the activity of *N*-acetoxy-2-acetylaminofluorene (Kapular and Michelson, 1971) or monofunctional nitrogen mustards (Price *et al.*, 1968) which react more readily with the heterocyclic bases in polynucleotides than with mononucleotides.

In considering the usefulness of BMB modified tRNAs in biologically relevant experiments it is pertinent to note that the tRNAs modified with a single fluorescent group can still be aminoacylated. Furthermore, the absorption spectrum of BMB has a peak at 325 nm (ϵ 4.1 × 10^4), well separated from the absorption of common nucleotides and quantum yield of BMB is high (0.11). However, the lifetime of the excited state of BMB is short (1.8 nsec) which limits its use by nanosecond fluorescence spectroscopy. Nevertheless, the short rotational correlation times observed in all three BMB-modified tRNA species examined (see also Yang and Söll, 1973b) indicate that BMB has a large extent of rotational freedom relative to the whole tRNA molecule.

The specificity of the reaction of BMB with tRNA offers a powerful tool for structural analysis of tRNA and its interactons in biological systems. It may be possible to design structural analogs of BMB retaining its specificity which have different optical characteristics (fluorophores with long excited-state lifetimes) or which can be used as spin labels or photoreactive labels for tRNA.

Acknowledgments

We are grateful to Drs. J. Carbon, A. D. Kelmers, N. J. Leonard, and G. Weimann for giving us valuable materials.

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