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# Design of New Photoaffinity Labels for Ribosomal Peptidyltransferase†

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ABSTRACT: The chemical syntheses of 6-azido-2′(3′)-*O*-L-phenylalanylpurine ribonucleoside (**4a**), 2′(3′)-*O*-(4-azido-L-phenylalanyl)adenosine (**4b**), and cytidylyl(3′→5′)-6-azido-2′(3′)-*O*-L-phenylalanylpurine ribonucleoside (**7**) are described. 6-Azidopurine ribonucleoside 5′-triphosphate (**10**) was also synthesized starting from 6-methylmercaptopurine ribonucleoside. All of these compounds (**4a, 4b, 7,** and **10**) are readily photolyzed by ultraviolet (UV) light. Compounds **4a, 4b,** and **7** are active in the ribosomal peptidyltransferase-catalyzed release of the Ac-Phe residue from the Ac-Phe-tRNA·70S ribosome·poly(U) complex. It follows that the 6-azidopurine moiety of compounds **4a** and **7,** as well as the 4-azido-L-phenylalanine moiety of **4b,** are recognized by the peptidyltransferase enzyme, and therefore these moieties are suggested for incorporation into tRNA as photoaffinity labeling reagents.

The affinity labeling method is a promising approach to the study of ribosomal structure. In principle, this method should yield information on the direct involvement of various elements (proteins and rRNAs) in ribosomal recognition sites. Thus, it should be possible to identify ribosomal components involved in the peptidyltransferase reaction, GTPase reaction, mRNA binding sites, etc. Until now most of the reagents used for affinity labeling of peptidyltransferase sites were analogues of *N*-acyl-AA-tRNA[1] or peptidyl-tRNA with an electrophilic or photoactive label attached to the *N*-acyl residue. Using various reagents of this type, several 50S proteins and 23S RNAs have been implicated as parts of the A and P sites of peptidyltransferase (for a review see Cooperman, 1977). To our knowledge, no labeling of peptidyltransferase sites with a functional analogue of AA-tRNA (possessing a free $\alpha$-amino group) has as yet been described.

In our approach to the affinity labeling of peptidyltransferase recognition sites we sought to develop labeling reagents with photoactive groups placed specifically at various positions of the 3′ terminus of AA-tRNA or Ac-AA-tRNA (e.g., on the 3′-terminal adenosine residue, or the amino acid moiety). Previous work with simple models of the 3′ terminus of AA-tRNA as substrates for the peptidyltransferase reaction has indicated that these entities play important roles in the binding of substrates to the enzyme loci. In addition, it was observed that peptidyltransferase can apparently tolerate considerable modification at position 6 of the adenosine residue (Rychlík et al., 1969, 1970; Žemlička et al., 1975; Chládek et al., 1976). We therefore decided to use as probes modified derivatives of AA-tRNA which have the photolabile azido group placed on the 3′-terminal nucleoside residue or on the amino acid side chain. In this report we describe the initial results of our approach utilizing 2′(3′)-*O*-aminoacyl nucleosides and oligonucleotides that incorporate an azido group. The chemical syntheses of several compounds of this type, containing 6-azidopurine ribonucleoside and 4-azidophenylalanine moieties, their behavior in the peptidyltransferase reaction, and their photolytic properties are described. In addition, we describe the synthesis of 6-azidopurine ribonucleoside 5′-triphosphate. This compound is designed to be incorporated enzymatically into the 3′ terminus of tRNA to yield a photoactive tRNA.

## Experimental Procedure

### *General Methods*

*Chromatography.* Paper chromatography was performed by the descending technique on Whatman No. 1 paper using the following solvent systems: $S_1$, 2-propanol-concentrated ammonium hydroxide-water (7:1:2); $S_2$, 1-butanol-acetic acid-water (5:2:3); $S_3$, butanol saturated with 10% acetic acid; $S_4$, 1-propanol-concentrated ammonium hydroxide-water (6:3:1); $S_5$, isobutyric acid-water-concentrated ammonia-0.1 M EDTA (57:38:4:1); $S_6$, 2-propanol-3 M ammonium hydroxide-0.1 M boric acid (7:2:1); $S_7$, ethanol-1 M ammonium acetate, pH 3.8 (5:2). Thin-layer chromatography (TLC) was done on silica gel coated aluminum foils (silica gel 60 F-254, Brinkmann Instruments, Westbury, N.Y.) in systems $S_8$

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[1] Abbreviations used are: AA-tRNA, aminoacyl transfer ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid (tetrasodium salt); $Et_3NH_2CO_3$, triethylammonium bicarbonate; DMF, dimethylformamide; $Me_2SO$, dimethyl sulfoxide; DCC, dicyclohexylcarbodiimide; DSS, 4,4-dimethyl-4-silapentane-5-sulfonate; Boc, *tert*-butyloxycarbonyl; A-Phe, 2′(3′)-*O*-L-phenylalanyladenosine; C-A-Phe, cytidylyl(3′→5′)-2′(3′)-*O*-L-phenylalanyladenosine; TLC, thin-layer chromatography; UV, ultraviolet; NMR, nuclear magnetic resonance.

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TABLE I: NMR Data for Products and Intermediates.[a]

| Compd | Multiplicity, chem shift (δ)[b] | | | | | | |
|---|---|---|---|---|---|---|---|
| | $H_2$ | $H_8$ | Aromatic protons | $H_{1'}$ | OH | Boc | $CH_3O$ |
| **2a** | s, 10.0 | s, 8.75 | m, 7.26<br>s, 6.85<br>s, 6.76 | d, 6.17 (4.2) | d, 5.69 (5.6)<br>d, 5.32 (5.4) | | s, 3.69 |
| **3a** | s, 10.1 | s, 8.85 | m, 7.29<br>s, 6.91<br>s, 6.82 | d, 6.20 (6.1) | | m, 1.36 | s, 3.53 |
| **3b** | s, 10.2 | s, 8.95 | s, 7.32 | d, 6.29 (6.4) | | m, 1.38 | |
| **3c** | s, 8.10 | s, 8.30 | m, 7.31<br>m, 7.22[c]<br>m, 7.07[c] | d, 5.96 (6.6) | | m, 1.36 | s, 3.75 |
| **3d** | s, 8.18 | s, 8.39 | q, 7.22[d] | d, 5.92 (6.8) | | m, 1.38 | |
| **4a** | s, 10.2 | s, 8.94 | s, 7.29 | | | | |
| **4b** | s, 8.19 | s, 8.38 | q, 7.25[d] | d, 5.94 (6.6) | | | |

[a] All spectra run in $CD_3SOCD_3$ with DSS as internal reference. [b] Coupling constants are shown in parentheses. [c] $a_2b_2$ system of 4-methoxy and 4-azidophenyl. [d] $a_2b_2$ system of 4-azidophenyl.

$[CHCl_3$–$CH_3OH$, 95:5], $S_9$ $[CHCl_3$–$CH_3OH$, 9:1], $S_{10}$ $[CH_3CN$–0.1 M $NH_4Cl$, 7:3], and $S_{11}$ [2-propanol–0.5 M $Et_3NH_2CO_3$, 9:2], and on PEI-cellulose (PEI/UV 254 cellulose MN 300, Brinkmann Instruments) in systems $S_{12}$ (1 M NaCl), $S_{13}$ (2 M NaCl), $S_{14}$ (0.75 M $KH_2PO_4$ adjusted to pH 3.8 with phosphoric acid).

Paper electrophoresis was conducted on a Savant flat plate apparatus using $E_1$ (1 M acetic acid), $E_2$ (0.02 M $Na_2HPO_4$, pH 7.0), $E_3$ (0.02 M $Et_3NH_2CO_3$, pH 7.5), $E_4$ (0.1 M $CH_3COONH_4$, pH 3.8), $E_5$ (0.05 M sodium hydrogen citrate, pH 3.5).

2′(3′)-*O*-Aminoacyl nucleosides and nucleotides were stored as stock solutions in 80% AcOH at −20 °C in the dark. Yields of nucleosides and nucleotides were determined spectrophotometrically using the following extinction coefficients: 6-methylmercaptopurine ribonucleoside, $\epsilon_{290}$ 20 200 (95% EtOH) (Žemlicka et al., 1975); 6-azidopurine ribonucleoside, $\epsilon_{287}$ 8200 (pH 1.0) (Johnson et al., 1958); 4-azidophenylalanine, $\epsilon_{252}$ 16 000 (water) (Schwyzer and Caviezel, 1971); and 2′(3′)-*O*-(4-azidophenylalanyl)adenosine, $\epsilon_{255}$ 28 600 (based on the $\epsilon_{255}$ for compound **3d**). All operations with azido derivatives were carried out in the dark. Other general methods were as described in previous papers (Chládek et al., 1974, 1977). NMR spectra were measured on a JEOL FX-100 in $Me_2SO$-$d_6$ using DSS as the internal standard. NMR data are listed in Table I.

*Photolysis Experiments.* Compounds were photolyzed using a 450-W Hanovia lamp (Ace Glass). The lamp was run at least 5 min prior to photolysis to allow the light output to stabilize. The sample concentrations (in water) are indicated in the figures. The solutions were maintained at 4 °C in a 3 mm i.d. cylindrical quartz cuvette during photolysis; at regular intervals aliquots were withdrawn, and after appropriate dilution with water, the UV spectra were recorded. Photolysis was done in a quartz cuvette with no additional filter, with a Pyrex filter (cutoff wavelength ca. 280 nm), or with a WG 320 Schott glass filter (cutoff 320 nm). Preliminary experiments indicated that purging by nitrogen bubbling had a negligible effect on the rate of photolysis.

Assay of peptidyltransferase activity was performed as described previously (Chládek et al., 1974) using the trichloroacetic acid precipitation method.

## *Materials*

5′-*O*-(4-Methoxy)trityladenosine was prepared according to the described procedure (Chládek and Zemlička, 1974), as were 4-azido-(*N*-*tert*-butyloxycarbonyl)-L-phenylalanine (Schwyzer and Caviezel, 1971), 6-azidopurine ribonucleoside (Wetzel and Eckstein, 1975), and 2′,5′-di-*O*-ditetrahydropyranylcytidine 3′-phosphate (Chládek et al., 1974). 4-Methoxytrityl chloride was purchased from Aldrich (Milwaukee, Wis.); 6-methylmercaptopurine ribonucleoside and carbonyldiimidazole were from Sigma (St. Louis, Mo.). Cytidylyl(3′→5′)adenosine was purchased from Calbiochem. Pyridine and DMF were treated as described (Chládek et al., 1977). All other reagents were distilled before use.

Ribosomes were prepared from late log phase *Escherichia coli* MRE 600 (RNase 1⁻) cells and were washed three times by ultracentrifugation in 0.5 M $NH_4Cl$ as described previously (Chládek et al., 1974). Ac[$^{14}$C]Phe-tRNA (0.4 nmol of [$^{14}$C]Phe/mg of tRNA) was prepared as described (Chládek et al., 1974).

*6-Azido-5′-O-(4-methoxy)tritylpurine Ribonucleoside* (**2a**). 6-Azidopurine ribonucleoside (**1**) (0.67 g, 2.57 mmol) was reacted with 4-methoxytrityl chloride (0.95 g, 3.08 mmol) by the method used for the analogous adenosine derivative (Chládek and Žemlička, 1974). The product was obtained by crystallization from chloroform (0.59 g, 41%) and shown to be pure by TLC ($S_5$, $R_f$ 0.65). A second crop of slightly contaminated product (0.72 g, 50%) was obtained from the mother liquor for a total yield of 91%. The UV spectrum (95% ethanol) showed: $\lambda_{max}$ 282 nm ($\epsilon$ 8680); $\lambda_{min}$ 260 nm ($\epsilon$ 6170); $\lambda_{sh}$ 230 nm ($\epsilon$ 21 500). Anal. Calcd for $C_{30}H_{27}N_7O_8$ (565.59): C, 63.70; H, 4.81; N, 17.34. Found: C, 63.54; H, 4.67; N, 17.42.

*6-Azido-5′-O-(4-methoxy)trityl-2′(3′)-O-(N-tert-butyloxycarbonyl-L-phenylalanyl)purine Ribonucleoside* (**3a**). Methoxytrityl derivative **2a** (0.566 g, 1.0 mmol) and *tert*-butyloxycarbonyl-L-phenylalanine (0.265 g, 1.0 mmol) were dissolved in dry pyridine (ca. 5 mL) and dried by repeated coevaporation with pyridine. The resulting mixture was then dissolved in pyridine (5 mL) and cooled to 0 °C with protection from moisture. With cooling and stirring, a cold solution of DCC (0.25 g, 1.2 mmol) in pyridine (ca. 2 mL) was added. The reaction mixture was stirred at 0 °C for 1 h, then at 25 °C for ca. 17 h. The reaction was quenched by the addition of ice (ca. 2 g), petroleum ether was added, dicyclohexylurea was filtered off, and the filtrate was washed with petroleum ether (3 × 25 mL). The washed pyridine fraction was rotary evaporated to a foam which was dissolved in dichloromethane, applied to two

loose layer silica gel plates, and developed in $S_8$ to give three prominent bands. The central band was collected, rechromatographed as before, and finally precipitated from petroleum ether to give a white powder in a yield of 0.218 g (49%). Purity was established by TLC ($S_8$, $R_f$ 0.58) and by paper chromatography ($S_2$, $R_f$ 0.86). The UV spectrum (95% EtOH) showed: $\lambda_{max}$ 282 and 262 nm; $\lambda_{min}$ 265 and 257 nm. Anal. Calcd for $C_{44}H_{44}N_8O_8$ (812.37): C, 65.01; H, 5.46; N, 13.79. Found: C, 64.57; H, 5.66; N, 13.79.

*6-Azido-2′(3′)-O-(N-tert-butyloxycarbonyl-L-phenylalanyl)purine Ribonucleoside* (**3b**). Compound **3a** (0.41 g, 0.5 mmol) was dissolved in 80% acetic acid and left at 25 °C for 17 h. The solution was then freeze dried, and the resulting residue coevaporated with absolute ethanol to dryness, dissolved in dichloromethane, and precipitated from petroleum ether to give a white powder in a yield of 0.21 g (78% from **3a**). The product **3b** was shown by TLC to be slightly contaminated. Further purification was achieved on a single silica gel loose layer plate (developed in $S_8$). After elution and precipitation with petroleum ether a white powder was obtained in a yield of 0.10 g (37% from **3a**). The resulting product **3b** was chromatographically pure on TLC ($S_8$, $R_f$ 0.11). The UV spectrum (0.01 N HCl) showed $\lambda_{max}$ 287 ($\epsilon$ 5650), 258 ($\epsilon$ 4150), 251 ($\epsilon$ 4570), and 243 nm ($\epsilon$ 4500), and $\lambda_{min}$ 265 ($\epsilon$ 3630), 256 ($\epsilon$ 4160), 247 ($\epsilon$ 4380), and 240 nm ($\epsilon$ 4450).

*6-Azido-2′(3′)-O-L-phenylalanylpurine Ribonucleoside* (**4a**). Compound **3b** (25 mg, 57 μmol) was dissolved in trifluoroacetic acid (1 mL) and left at 25 °C. After 15 min the solution was rotary evaporated to dryness and coevaporated with dioxane (3×). The resulting residue was dissolved in dioxane, precipitated with petroleum ether to give an oil, collected by centrifugation, and washed with petroleum ether (3×). After dissolving the resulting oil in 80% acetic acid, the spectrophotometrically determined yield was 14.2 μmol (25% from **3b**, based on the $\epsilon$ at 287 nm for the parent nucleoside). The UV spectrum (0.01 N HCl) gave $\lambda_{max}$ 287, 259, and 251 nm, and $\lambda_{min}$ 265, 255, and 247 nm (the fourth peak seen in **3b** was apparently obscured by acetic acid absorbance). The ninhydrin positive product **4a** was checked by electrophoresis ($E_1$, mobility 0.84 relative to adenosine), paper chromatography ($S_2$, $R_f$ 0.69; adenosine, $R_f$ 0.49), and NMR spectra.

*2′(3′)-O-[4-Azido-(N-tert-butyloxycarbonyl)-L-phenylalanyl]-5′-O-(4-methoxy)trityladenosine* (**3c**). Compound **2b** (0.54 g, 1.0 mmol) and 4-azido-(*N-tert*-butyloxycarbonyl)-L-phenylalanine (0.31 g, 1.0 mmol) were condensed in pyridine with DCC as for **3a** above. After workup, the resulting mixture was chromatographed on two silica gel loose layer plates ($S_9$) to give three prominent bands. The central (product) band was collected, rechromatographed on a single loose layer plate (developed in $S_8$), collected, and precipitated from petroleum ether. The yield of **3c** was 0.28 g (34%) as a white powder.

The compound was chromatographically uniform on silica gel TLC ($S_9$, $R_f$ 0.63). The UV spectrum (95% ethanol) showed: $\lambda_{max}$ 255 nm; $\lambda_{sh}$ 290 and 240 nm; $\lambda_{min}$ 227 nm; 250/260 = 0.98, 280/260 = 0.32, 290/260 = 0.12. Anal. Calcd for $C_{44}H_{45}N_9O_8 \cdot 0.5H_2O$ (836.9): C, 63.14; H, 5.54; N, 15.06. Found: C, 62.92; H, 5.54; N, 15.15.

*2′(3′)-O-[4-Azido-(N-tert-butyloxycarbonyl)-L-phenylalanyl]adenosine* (**3d**). Compound **3c** (0.1 g) was dissolved in 80% acetic acid (5 mL), left at 25 °C for 17 h, and then freeze-dried. The resulting residue was dissolved in dichloromethane and applied to a single loose layer silica gel plate (developed in $S_9$). The major band was eluted and precipitated from petroleum ether to give **3d** in a yield of 35.9 mg (53%) of white powder. TLC ($S_8$) of **3d** gave a single spot ($R_f$ 0.45). The UV spectrum (5% methanol/0.01 N HCl) showed: $\lambda_{max}$ 255 nm ($\epsilon$ 28 600); $\lambda_{min}$ 227 nm; 250/260 = 1.02, 280/260 = 0.23, 290/260 = 0.10.

*2′(3′)-O-[4-Azido-L-phenylalanyl]adenosine* (**4b**). Compound **3d** (20 mg, 36 μmol) was dissolved in freshly distilled trifluoroacetic acid (0.5 mL), left for 5 min, and immediately evaporated under vacuum, followed by several coevaporations with dioxane. The resulting residue was dissolved in 80% acetic acid and the yield was determined spectrophotometrically (18.5 μmol, 51%, based on $\epsilon_{255}$ for **3d**). The purity of the product was confirmed by NMR spectra, paper electrophoresis ($E_1$, mobility 1.2 relative to adenosine), and by TLC ($S_2$, single spot $R_f$ 0.73); the spots in both cases were ninhydrin positive. The UV spectrum (0.01 N HCl) showed: $\lambda_{max}$ 255 nm; $\lambda_{min}$ 226 nm; 250/260 = 1.06, 280/26] = 0.22, 290/260 = 0.80.

*Cytidylyl-(3′→5′)-6-azido-2′(3′)-O-(N-tert-butyloxycarbonyl-L-phenylalanyl)purine ribonucleoside* (**6**). Compound **3b** (64 mg, 118 μmol) was condensed with 2′,5′-di-*O*-tetrahydropyranyl-*N*-dimethylaminomethylenecytidine 3′-phosphate (**5**) (0.1 mmol) by the method of Chládek et al. (1974), except that the reaction mixture was protected from light during reaction and chromatography. After deblocking in the usual manner, the product **6** was chromatographed on two preparative cellulose TLC plates ($S_3$). The central (product) band was eluted with $S_2$ and rotary evaporated, and the resulting residue was dissolved in methanol/trace acetic acid. The yield (15.5 μmol, 13%) was determined spectrophotometrically in 0.01 N HCl using a (calculated) value of 18 400 for $\epsilon$ at 287 nm. Chromatography of the product **6** on cellulose TLC ($S_3$, $R_f$ 0.47; adenosine, $R_f$ 0.36), and paper electrophoresis ($E_1$, mobility of 0.07 relative to adenosine) showed that the product was not completely pure; however, in anticipation of final deblocking, no further purification of this product was attempted. The UV spectrum (0.01 N HCl) showed: $\lambda_{max}$ 284 nm; $\lambda_{sh}$ 262 and 253 nm; $\lambda_{min}$ 245 nm; 250/260 = 0.79, 280/260 = 1.49, 290/260 = 1.38.

*Cytidylyl(3′→5′)-6-azido-2′(3′)-O-L-phenylalanylpurine Ribonucleoside* (**7**). With protection from light, an aliquot of compound **6** (10 μmol) was rotary evaporated to dryness, dissolved in trifluoroacetic acid (0.5 mL), and allowed to react at 25 °C for 5 min. The solution was immediately evaporated to dryness, dissolved in 80% acetic acid, and applied to Whatman No. 3MM paper for preparative paper electrophoresis in $E_1$ (4000 V for 3.5 h). The major (product) band was cut out, eluted with $S_2$, evaporated to dryness, and dissolved in 80% acetic acid (protected from light during electrophoresis and elution). The spectrophotometrically determined yield was 2.96 μmol. Paper electrophoresis ($E_1$) showed a ninhydrin positive spot (mobility 0.6 relative to adenosine), with a very slight ninhydrin positive contamination corresponding to phenylalanine. The UV spectrum (0.01 N HCl) showed: $\lambda_{max}$ 280 nm; $\lambda_{sh}$ 258 and 252 nm; $\lambda_{min}$ 247 nm; 250/260 = 0.84, 280/260 = 1.40, 290/260 = 1.15.

*Pancreatic Ribonuclease Digest of* **7**. Due to the lability of **7** at high pH, special conditions had to be employed. It was first determined (by UV spectra) that the 6-azidopurine moiety of **7** was stable when **7** was incubated at 37 °C in 0.1 N Tris-HCl buffer (adjusted to pH 7.3 at 4 °C). It was then demonstrated that authentic C-A (0.34 mmol/0.1 mL of buffer) was completely digested by pancreatic ribonuclease (1 mg/mL of buffer) when incubated in the above buffer for 30 min at 37 °C.

A sample of **7** (0.25 μmol in 0.1 mL of buffer) was incubated under the same conditions as C-A above. The incubation mixture was separated by paper electrophoresis on Whatman

No. 3MM paper ($E_2$, 2 h at 4000 V). The spots were cut out and eluted with 0.01 N HCl and quantitated spectrophotometrically. The ratio of Cp/6-azidopurine ribonucleoside was found to be 1.2, and the amount of unsplit material 7.2%.

*6-Methylmercaptopurine Ribonucleoside 5′-Monophosphate* (**8b**). A suspension of 6-methylmercaptopurine ribonucleoside (**8a**; 0.9 g, 3 mmol) in triethyl phosphate (7.5 mL) was cooled with an ice bath. Dropwise addition of $POCl_3$ (0.56 mL, 6 mmol) with stirring gave a clear solution in approximately 1 h. Stirring was continued for an additional hour after which the excess of $POCl_3$ was evaporated in vacuo. The solution was cooled and neutralized with 2 M $Et_3NH_2CO_3$ (15 mL), evaporated in vacuo, and coevaporated three times with water. The milky residue was dissolved in aqueous 0.13 M $Et_3NH_2CO_3$ and the solution applied to a DEAE-cellulose column (3.5 × 55 cm) preequilibrated with 0.13 M $Et_3NH_2CO_3$. The column was eluted at 4 °C with a linear gradient of 0.13 (2 L) to 0.2 M $Et_3NH_2CO_3$ (2 L). Fractions were collected (ca. 20 mL/10 min), with the desired product **8b** eluting in fractions 70–100. These fractions were evaporated and the residue was coevaporated several times with methanol. The residue was dissolved in methanol and the yield was determined spectrophotometrically (1.4 mmol, 47%). The nucleotide was found to be pure chromatographically ($S_4$, $R_f$ 0.37; $S_5$, $R_f$ 0.90; $S_{13}$, $R_f$ 0.65) and electrophoretically ($E_4$, mobility 1.81 relative to AMP; $E_5$, mobility 1.89 relative to AMP). Compound **8b** also moved as a single spot in system $S_6$. The sodium salt of **8b** was prepared as described (Chládek et al., 1977) and had a molecular weight of 554 (determined spectrophotometrically in water). The UV spectrum (MeOH) showed: $\lambda_{max}$ 283 nm; $\lambda_{sh}$ 289 nm; 250/260 = 0.62, 270/260 = 2.07, 280/260 = 3.86, 290/260 = 3.93.

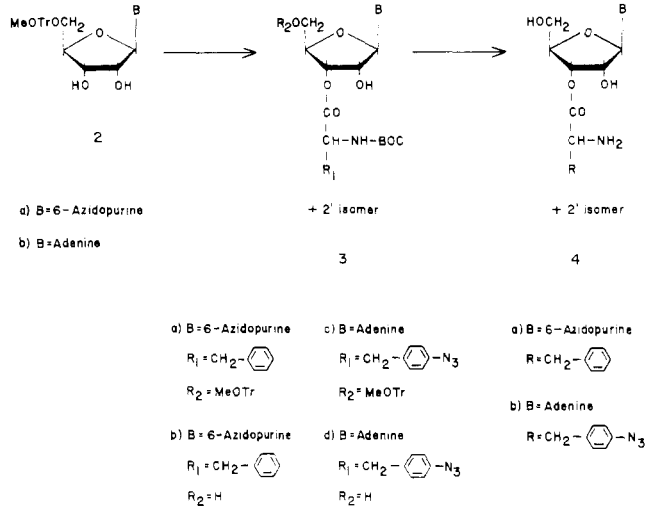
*6-Methylmercaptopurine Ribonucleoside 5′-Triphosphate* (**8c**). The monophosphate **8b** (0.7 mmol) was dried by coevaporation with anhydrous pyridine (3×) and the resulting glass was coevaporated with DMF (2×) and dissolved in DMF (4 mL). *N,N′*-Carbonyldiimidazole (0.23 g, 1.4 mmol) was added. After 1 h of reaction time, TLC ($S_{11}$) showed the quantitative conversion of starting material **8b** to the faster moving imidazolide. After 1.5 h total reaction time, tetrakis(tri-*n*-butylammonium) pyrophosphate (5 mmol) (Faerber and Scheit, 1971) in $Me_2SO$ (7 mL) was added and the reaction mixture (containing an oily precipitate) was stirred overnight at room temperature. TLC ($S_{12}$) of the clear solution showed quantitative conversion to triphosphate **8c**. The reaction mixture was diluted with 0.2 M $Et_3NH_2CO_3$ and applied to a column of DEAE-cellulose (3.5 × 55 cm) at 4 °C. The column was washed with 0.2 M $Et_3NH_2CO_3$ until $Me_2SO$ was removed. A linear gradient of 0.2 (2 L) to 0.45 M (2 L) $Et_3NH_2CO_3$ was used to elute the triphosphate **8c**, which appeared in fractions 60–120 (20 mL/10 min). The eluate was evaporated, and the residue coevaporated with methanol. The yield (determined spectrophotometrically) was 0.6 mmol (88%) of chromatographically ($S_4$, $R_f$ 0.21; $S_5$, $R_f$ 0.40; $S_{13}$, $R_f$ 0.33) and electrophoretically ($E_4$, mobility 2.68 relative to AMP; $E_5$, mobility 3.13 relative to AMP) uniform substance **8c**. The UV spectrum (95% ethanol) showed: $\lambda_{max}$ 290 nm ($\epsilon$ 18 900); $\lambda_{sh}$ 287 nm; $\lambda_{min}$ 248 nm; 250/260 = 0.56, 270/260 = 1.92, 280/260 = 3.76, 290/260 = 4.56. The $Na^+$ salt of **8c** was prepared according to Chládek et al. (1977). Anal. Calcd for $C_{11}H_{13}N_4O_{13}P_3SNa_4 \cdot 4H_2O$ (698.27): P, 13.31; N, 8.02; S, 4.59. Found: P, 13.44; N, 8.25; S, 4.12.

*6-Azidopurine Ribonucleoside 5′-Triphosphate* (**10**). The triethylammonium salt of **8c** (0.35 mmol) was dissolved in 0.2 M Tris-HCl (pH 7.5, 35 mL) and *N*-chlorosuccinimide (0.49 g, 3.5 mmol) was added. After 20 min of stirring at room temperature the UV spectrum of the reaction mixture (in water) showed a spectral shift from $\lambda_{max}$ 290 nm for **8c** to $\lambda_{max}$ 278 nm for the sulfone (**9**). Pyridine (14 mL) was added and the solution was stirred for 20 min. The solution was evaporated and then coevaporated with dioxane and methanol. The residue was dissolved in anhydrous methanol (15 mL) and a solution of dry sodium azide (0.14 g, 2.1 mol) in methanol (15 mL) was added; a pink precipitate formed immediately. The mixture was shaken at room temperature until the UV spectral ratios (see below) were constant (4 days). The solution was diluted with water (150 mL) and applied to the top of a column of DEAE-Sephadex A-25 (1.3 × 57 cm) in the chloride form (previously equilibrated with water). Chromatography was performed at 4 °C with the exclusion of light. The products were eluted with a linear gradient of 0.1 M LiCl in water (1 L) to 0.35 M LiCl in 0.05 M formic acid (1 L). Fractions of 10 mL were collected (flow rate ca. 0.2 mL/min). The desired triphosphate **10** was found in fractions 55–70, immediately preceded by a substantial contaminant. The fractions of **10** were freeze-dried and coevaporated several times with dioxane and then with anhydrous acetone. The white solid was repeatedly extracted (at 4 °C) by stirring with anhydrous acetone until inorganic salts were no longer detectable by the silver nitrate test. A solid material was obtained by centrifugation in anhydrous acetone. The yield of chromatographically ($S_{12}$, $R_f$ 0.03; $S_{14}$, $R_f$ 0.31) and electrophoretically ($E_5$, mobility 3.07 relative to AMP) homogeneous $Li^+$ salt of **10** was 0.11 g (43%). The UV spectrum (0.01 N HCl) showed: $\lambda_{max}$ 287 ($\epsilon$ 7950), 260 ($\epsilon$ 4460), and 252 nm ($\epsilon$ 4360); $\lambda_{min}$ 265, 256, and 245 nm; 250/260 = 0.94, 270/260 = 1.11, 280/260 = 1.62, and 290/260 = 1.76 are in perfect agreement with ratios for the described crystalline nucleoside **1** (Wetzel and Eckstein, 1975). Anal. Calcd for $C_{10}H_{10}N_7O_{13}P_3Li_4 \cdot 7H_2O$ (683.8): P, 13.59; N, 14.34. Found: P, 13.59; N, 14.40.

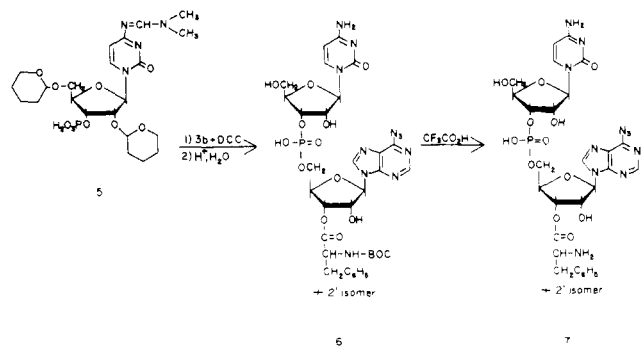
## Results and Discussion

In order to develop a new photolabile species of AA-tRNA for cross-linking experiments with ribosomes or elongation factor $T_u$, it is necessary to enzymatically incorporate the modified photolabile nucleosides into the 3′ terminus of tRNA or to affect the enzymatic aminoacylation of the native tRNA with photolabile analogues of an amino acid. As a prerequisite, it was important to establish whether or not these modified moieties are recognized by, e.g., peptidyltransferase. Since this enzyme is known to utilize simple 2′(3′)-*O*-aminoacyl nucleosides as acceptor substrates (Rychlík et al., 1969, and references cited therein), the easiest test of the feasibility of the proposed modifications was to synthesize the appropriate modified 2′(3′)-*O*-aminoacyl nucleosides and investigate them in peptidyltransferase assay systems before any work on the tRNA level was undertaken. At present we have considered two modifications of the basic skeleton of A-Phe (as the simplest acceptor substrate): substitution on the purine ring by the azido group at position 6, and addition of the azido group to the benzene ring of phenylalanine. In choosing the azido group we have taken into consideration not only its photolability, but also the factor of length. This group is short (2.4 Å) relative to most of the functions used previously for the affinity labeling of peptidyltransferase centers on ribosomes (Cooperman, 1977). Thus, the presence of the azido group at the 3′ terminus of AA-tRNA should result in only minimal distortion at the enzymatic site. Moreover, and perhaps of greater significance, the photoproduced nitrene (length ca. 1.5 Å) should react specifically with ribosomal elements which are a part of the binding site at the terminus of AA-tRNA, rather than with elements which are only in the general vicinity, thus affording

SCHEME I



SCHEME II



a better resolution than that obtained with previous labels.

Accordingly, we have synthesized 6-azido-2′(3′)-*O*-L-phenylalanylpurine ribonucleoside (**4a**) and 2′(3′)-*O*-(4-azido-L-phenylalanyl)adenosine (**4b**) by established procedures (Chládek et al., 1970). 6-Azidopurine rubonucleoside (**1**)[2] was first converted to its 5′-*O*-(4-methoxytrityl) derivative (**2a**), which was then aminoacylated with Boc-Phe and DCC in pyridine solution to yield the monoaminoacylated derivative **3a** in 49% yield after purification by TLC (Scheme I). The 4-methoxytrityl group was selectively removed by treatment with acetic acid; it was ascertained that the Boc group is fully stable under the conditions employed. The slightly contaminated **3b** which resulted was isolated in 78% yield.[3] Finally, the unprotected 6-azido-2′(3′)-L-phenylalanylpurine ribonucleoside (**4a**) was obtained after removal of the Boc group with trifluoroacetic acid. The second analogue of A-Phe, 2′(3′)-*O*-(4-azido-L-phenylalanyl)adenosine (**4b**), was derived in a similar way starting from 5′-*O*-(4-methoxytrityl)adenosine (**1b**) and 4-azido-*N*-(*tert*-butyloxycarbonyl)-L-phenylalanine.

The photolabile analogue of C-A-Phe (which is the sequence of the terminus of Phe-tRNA), in which the adenosine moiety is replaced by 6-azidopurine ribonucleoside (**1**), was synthesized by the general route for the synthesis of 2′(3′)-*O*-aminoacyl derivatives of dinucleoside phosphates (Chládek et al., 1974; Chládek and Žemlička, 1974) (Scheme II). Since all protecting groups used for the synthesis must be removable in acidic medium (to preserve the integrity of the aminoacyl group), *N*-dimethylaminomethylene-2′,5′-di-*O*-tetrahydropyranylcytidine 3′-phosphate (Chládek et al., 1974) was used as the nucleotide component for the DCC-mediated condensation with **3b.** Therefore, the only procedural difference from earlier work (Chládek et al., 1974) was the use of the Boc group for protection of the amino acid moiety, rather than the *N*-benzyloxycarbonyl group. The latter group is clearly incompatible with the 6-azido group, due to the necessity of its removal by hydrogenation. In a previous test we found that C-A is partly hydrolyzed by treatment with trifluoroacetic acid (ca. 10%), but quite surprisingly its phosphodiester linkage is not isomerized to any significant degree, as assayed by cleavage with pancreatic ribonuclease (see also Mercer and Symons, 1972). Accordingly, we achieved deprotection of the condensation product in the following manner: first, treatment with chromatographic system $S_2$ to remove the *N*-dimethylaminomethylene group, then with 0.05 N HCl to remove the tetrahydropyranyl groups (these conditions did not cause loss of the Boc group). These two steps were followed by preparative TLC of compound **6** on cellulose in acidic medium. After removal of the Boc group by brief treatment with trifluoroacetic acid, the final reaction product, cytidylyl(3′→5′)-6-azido-2′(3′)-*O*-L-phenylalanylpurine ribonucleoside (**7**), was isolated by preparative paper electrophoresis. The nearly quantitative cleavage (93%) of the final product by pancreatic ribonuclease shows the relatively minor isomerization of the acid-sensitive 3′→5′ phosphodiester linkage of **7** during deblocking and isolation procedures.

Studies of the enzymatic synthesis (using tRNA nucleotidyltransferase; Deutscher, 1973) of modified tRNA with the 6-azidopurine moiety incorporated at the 3′ terminus of tRNA require 6-azidopurine ribonucleoside 5′-triphosphate. We attempted to approach this problem via phosphorylation of the readily available 6-azidopurine ribonucleoside (**1**) (Wetzel and Eckstein, 1975) by $POCl_3$ in triethyl phosphate (Yoshikawa et al., 1967), which was to be followed by the formation of a triphosphate linkage through one of the standard methods. It was quickly discovered, however, that the 6-azidopurine moiety is extremely unstable in the mild alkaline medium required for the isolation of 6-azidopurine ribonucleoside 5′-monophosphate. We found (Figure 1) that 6-azidopurine ribonucleoside decomposes on holding in aqueous 0.5 M $Et_3NH_2CO_3$ at 4 °C. This decomposition can be observed spectrophotometrically as a decrease of UV absorption at 287 nm; the absorption is completely lost after 47 h. The products of this reaction were not identified, but the loss of UV absorption indicates cleavage of all rings in the base moiety of **1.** It is interesting to note that 1,$N^6$-ethenoadenosine, which is related to the tetrazole form of **1,** is also alkali labile (Barrio et al., 1972). The instability of 6-azidopurine ribonucleoside in strongly alkaline systems was noted earlier (Johnson et al., 1958; Temple et al., 1966). It was also observed (Temple et al., 1966) that in alkaline media the tetrazole form **1b** (Scheme III) is first cleaved at the pyrimidine ring (A) resulting in the imidazol-5-yltetrazole which is also unstable over a wide pH range. 6-Methylmercaptopurine ribonucleoside 5′-monophosphate (**8b**) was synthesized by the procedure of Yoshikawa et al. (1967) and isolated in 47% yield by ion-exchange chromatography[4]

[2] 6-Azidopurine ribonucleoside is known to exist in equilibrium with the tetrazole form, with the latter prevailing (Johnson et al., 1958; Temple et al., 1966) (Scheme IV).

[3] Purification of this product on TLC of silica gel led to large losses of material. It has been reported that the azide-tetrazole equilibrium is shifted toward the azido form in acidic medium (Temple et al., 1966). Thus, it is possible that due to acidic conditions during silica gel chromatography, photodecomposition of compound **3b** may have occurred. This may also explain the low yields of other 6-azidopurine derivatives which were treated in acidic media.

[4] Compound **8b** was described previously, prepared via phosphorylation of the 2′,3′-*O*-isopropylidine derivative of **8a** by β-cyanoethyl phosphate and DCC (Pfleiderer et al., 1964).

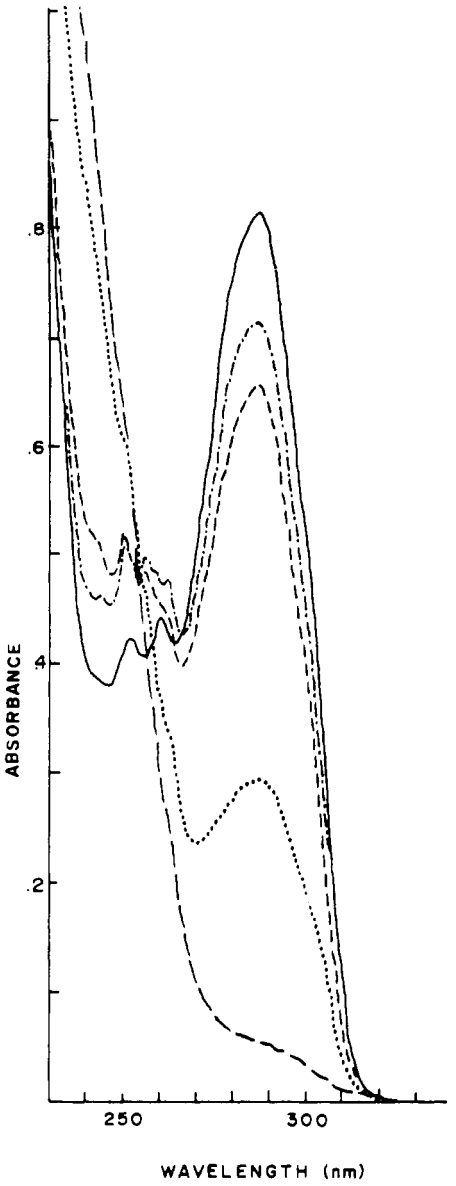
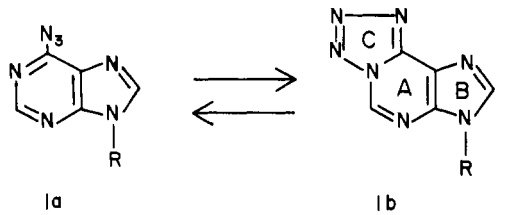
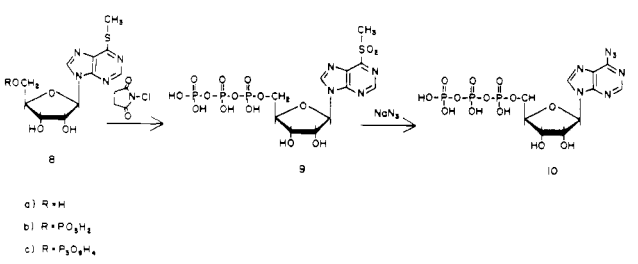



FIGURE 1: Decomposition of 6-azidopurine ribonucleoside (**1**) in aqueous $Et_3NH_2CO_3$. Compound **1** was dissolved in 0.5 M $Et_3NH_2CO_3$ (pH 7.5) (compound concentration 1 mM) and kept at 4 °C. UV spectra were measured at the times shown: (—) 0 h; (- · -) 1 h; (- - -) 4 h; (· · ·) 22 h; (– – –) 47 h.

SCHEME III




SCHEME IV



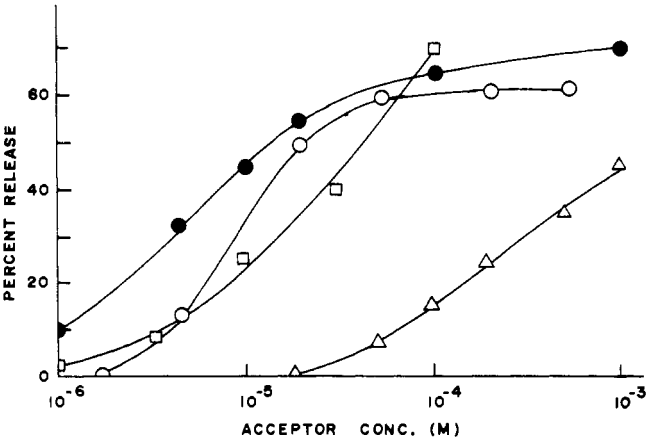




FIGURE 2: Acceptor-dependent release of the Ac[$^{14}$C]Phe residue from Ac[$^{14}$C]Phe-tRNA in the peptidyltransferase reaction. Percent release represents the acceptor-dependent decrease of $CCl_3COOH$ precipitated counts trapped by Millipore membrane. For other details see Chládek et al. (1974); (△) **4a**; (□) **7**; (○) **4b**; (●) A-Phe.

(Scheme IV). The triphosphate derivative **8c** was prepared from compound **8b** via activation with carbonyldiimidazole and subsequent reaction with tributylammonium pyrophosphate (Faerber and Scheit, 1971). The product **8c** was isolated by ion-exchange chromatography in 88% yield. Although the facile conversion of 6-methylmercaptopurine ribonucleoside to the corresponding sulfone by oxidation with chlorine in methanol solution has been described (Wetzel and Eckstein, 1975), it was assumed that these conditions would not be suitable for the analogous reaction with the triphosphate **8c.** Instead we achieved the oxidation with *N*-chlorosuccinimide. The sulfone **9** was used immediately in the subsequent reaction with sodium azide because we found that it was too unstable to be isolated in pure form. The quantitative displacement of sulfone **9** with sodium azide was achieved after ca. 4 days at room temperature. The final product **10** was isolated by column chromatography on DEAE-Sephadex in acidic medium in 43% yield and was fully characterized.

Compounds **4a, 4b,** and **7** were investigated as acceptor substrates in the peptidyltransferase reaction. In light of the aforementioned lability of the 6-azidopurine moiety in weakly alkaline medium, it was first ascertained that this moiety is fully stable under the conditions of the peptidyltransferase assay. Figure 2 shows that compound **4a** was moderately active in release of Ac-Phe residue from the Ac-Phe-tRNA·70S ribosome·poly(U) complex. Its activity is somewhat lower than that of 2′(3′)-*O*-L-phenylalanyl-1,$N^6$-ethenoadenosine (Chládek et al., 1976) which is isosteric with the tetrazole form of **4a** (Scheme III; Temple et al., 1966; Johnson et al., 1958). The significant decrease in acceptor activity with **4a,** relative to the unmodified A-Phe, may be caused by an unfavorable influence of the tetrazole ring fused in positions 1 and 6 of the adenine residue. An alternative explanation is nonproductive binding (Zeeberg et al., 1975) of the tetrazole and pyrimidine rings (rings A and C, Scheme III) to the appropriate enzymic sites relative to productive binding (rings A and B) of the natural adenosine. The considerable increase in acceptor activity for the dinucleotide derivative **7** compared to that of nucleoside **4a** is in agreement with the stimulatory effect on acceptor activity of joining the cytidine 3′-phosphate residue to 2′(3′)-*O*-aminoacyladenosines (Rychlík et al., 1967). It is thus felt that biologically active species of the modified tRNA can be constructed by incorporating the 6-azidopurine residue into the 3′ terminus of tRNA.

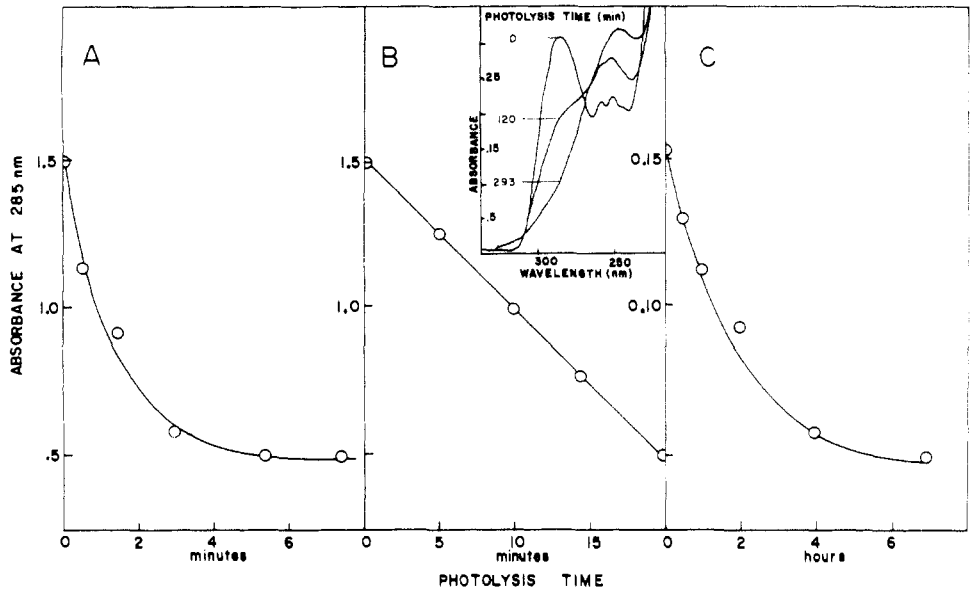



FIGURE 3: Rate of photolysis of **4a** (2 mM) measured as a function of changing absorbance at 285 nm: (A) quartz filter; (B) Pyrex filter; (C) WG 320 filter. The inset of B shows spectral changes during irradiation of **4a**.

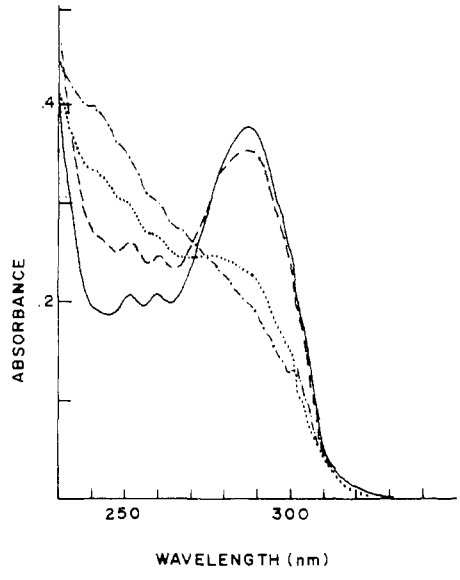



FIGURE 4: Spectral changes during irradiation of **10** (0.64 mM) with WG 320 filter: (—) 0 h; (- - -) 1 h; (· · ·) 3 h; (- · -) 4 h.

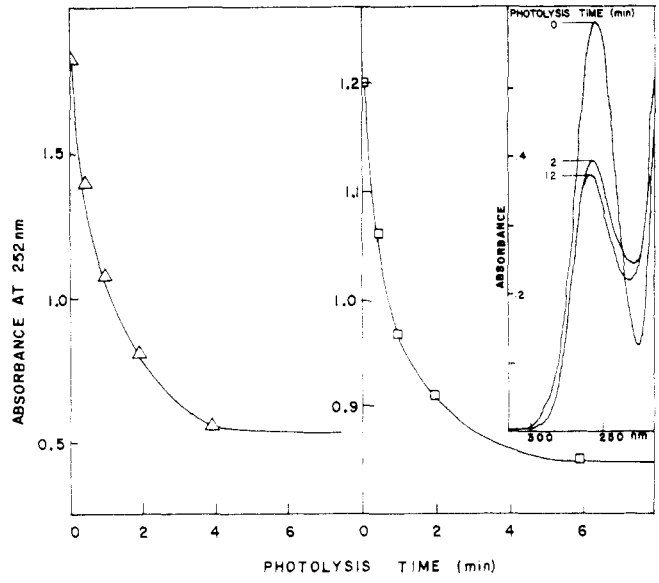



FIGURE 5: Rate of photolysis measured as a function of changing absorbance at: (a, left) 252 nm (△, 4-azidophenylalanine, 12 mM) and (b, right) 260 nm (□, **4b**, 4 mM) with WB 320 filter. The inset of b shows spectral changes during irradiation of **4b**.

As can also be seen from Figure 2, the acceptor activity of **4b** is very similar to that of unmodified A-Phe. Thus, the addition of the relatively short azido group to the aromatic portion of phenylalanine interferes only slightly with the normal activity of A-Phe in the peptidyltransferase reaction. The existence of a binding hydrophobic locus for the amino acid side chain of AA-tRNA within the peptidyltransferase A site has been postulated (Rychlík et al., 1970); this locus should be able to accommodate various amino acid side chains, including the side chain of 4-azido-L-phenylalanine, which may be regarded as a tyrosine analogue (Schwyzer and Caviezel, 1971).

The photolytic properties of the potential affinity labels were investigated in detail. It was noted earlier that 6-azidopurine ribonucleoside (**1**) undergoes slow photodecomposition (Wetzel and Eckstein, 1975). 4-Azidophenylalanine is also photolabile (Escher and Schwyzer, 1974) and was used (as an N-terminal amino acid residue in dipeptidyl-tRNA) for affinity labeling of ribosomes by irradiation with wavelengths above 250 nm (Sonenberg et al., 1975). We have found with our apparatus (Figure 3) that compound **4a** is readily photolyzed by UV irradiation, as evidenced by spectral changes, with $t_{1/2}$ = 1.2 min at wavelengths above 254 nm and $t_{1/2}$ = 8.5 min at wavelengths above 280 nm. Photolysis conducted above 320 nm is much slower ($t_{1/2}$ = 3.4 h). It was ascertained that the photolytic conditions necessary for complete decomposition of **4a** (using 280-nm cutoff) do not damage ribosomes, as established by peptidyltransferase assay of preirradiated ribosomes (results not shown). The spectral changes of the triphosphate derivative **10** as a result of photolysis are shown in Figure 4. Figures 5a and 5b show the rate of photolysis of 4-azidophenylalanine and its adenosine ester **4b**; these measurements were obtained from the UV spectral data using a 320-nm cutoff. It can be seen that half-times ($t_{1/2}$) are 0.85 min for 4-azidophenylalanine and 0.6 min for **4b**. We have observed that these conditions are also not detrimental to ribosomes, determined as above.

A photoaffinity labeling experiment was attempted in which Ac-[$^{14}$C]Phe-tRNA·70S ribosome·poly(U) complex was irradiated in the presence of nonradioactive **4a** (concn 5 mM), in the hope that covalently bound (via photoreaction) **4a** would undergo the peptidyltransferase reaction and thus radioactively label the 70S ribosome. In our preliminary experiments we were able to demonstrate only very weak labeling of the 50S subunit by this rather insensitive method (results not shown). Therefore, we made no further experiments along this line with any other labels. Since aminoacyl nucleosides (e.g., puromycin) bind very weakly to ribosomes ($K_m$ of puromycin = 125 $\mu$M) (Hussain and Ofengand, 1972) this emphasizes the necessity of using modified tRNAs for affinity labeling experiments.

In summary, the photolytic and enzymatic properties of modified 2′(3′)-*O*-aminoacyl nucleosides **4a** and **4b** appear to warrant further efforts to accomplish the incorporation of 6-azidopurine ribonucleoside and 4-azidophenylalanine moieties into AA-tRNA as the 3′-terminal nucleoside or aminoacyl residues, respectively. It is expected that such modified AA-tRNAs will be useful as labeling reagents in the study of ribosomal structure.

## Note Added in Proof

Since the completion of this research we have learned that Dr. B. S. Cooperman and coworkers (University of Pennsylvania) synthesized 3′-deoxy-3′-(*p*-azido-L-phenylalanylamido)-6-dimethyladenosine (similar to our compound **4b**) and used it for affinity labeling of ribosomes (personal communication).

## Acknowledgments

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