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Synthesis and Characterization of 5-[(4-Azidophenacyl)thio]uridine 5′-Triphosphate, a Cleavable Photo-Cross-Linking Nucleotide Analogue†

Michelle M. Hanna,* Suzanne Dissinger, Bradley D. Williams, and James E. Colston

Department of Biological Chemistry, California College of Medicine, University of California, Irvine, Irvine, California 92717

Received January 30, 1989; Revised Manuscript Received March 23, 1989

ABSTRACT: The synthesis, isolation, and characterization of a new photo-cross-linking uridine 5′-triphosphate analogue are described. This nucleotide analogue, 5-[(4-azidophenacyl)thio]uridine 5′-triphosphate (5-APAS-UTP), contains an aryl azide group approximately 10 Å from the uridine ring. The azide is photoactivated by irradiation at 300 nm, resulting in covalent attachment of the nucleotide to adjacent molecules. The nucleotide can be desulfurated with Raney nickel to cause molecular cleavage between the base and the aryl azide. Desulfuration yields uridine 5′-triphosphate and *p*-azidoacetophenone. If the analogue is cross-linked to another molecule, desulfuration leaves the analogue's acetophenone group attached to that molecule. This effectively leaves behind a molecular tag on molecules that interact with the uridine analogue either as monomeric nucleotide or as part of an RNA molecule. This nucleotide analogue can be incorporated into internal positions in RNA by transcription in vitro with *Escherichia coli* RNA polymerase. It can therefore be used to examine interactions between RNA and other molecules (e.g., proteins or nucleic acids). Because the sulfur atom can be selectively removed, the covalent bonds formed between analogue-containing RNA and other molecules can be cleaved, when desired, to facilitate identification of the cross-linked molecules and RNA nucleotides in the cross-linked complex.

Photochemical cross-linking is a powerful tool for the characterization of RNA–protein or RNA–nucleic acid interactions that occur during biological processes. Although there are a number of approaches one can take to photo-cross-linking of RNA, one of the most selective involves the incorporation of nucleotide analogues containing photoreactive cross-linking groups into the RNA molecule. Irradiation with ultraviolet or visible light converts these chemically inert groups to chemically reactive species that can covalently bond to adjacent molecules.

A number of nucleotide analogues have been characterized that have different cross-linking groups, excitation maxima, distances between the nucleotide and the cross-linker, and cross-linker position on the nucleotide (base, sugar, or phosphate) [for review, see Hanna (1988)]. Many of these contain photoreactive aryl azides as the cross-linking functional group. The half-life of the photolytically produced reactive species, the nitrene, is on the order of a millisecond, and its insertion reactions are highly nonspecific (Knowles, 1972; Bayley & Knowles, 1977; Schrock & Schuster, 1984). This nonspecificity is particularly useful when the environment of a protein or nucleic acid is probed, as there need not be a specific functional group within contact of the nitrene to get cross-linking.

Two types of azide-tagged nucleotide analogues containing cleavable bonds function as transcription initiation substrates for *Escherichia coli* RNA polymerase. These have been used to examine the interactions between the 5′ end of an RNA molecule and the RNA polymerase catalyzing its synthesis (Grachev & Zaychikov, 1981; Hanna & Meares, 1983a,b; Bernhard & Meares, 1986a,b; Stackhouse & Meares, 1988). However, many molecular interactions between RNA molecules and proteins or nucleic acids involve internal RNA sequences that can only be probed by incorporation of photo-cross-linking nucleotides within the RNA chain.

Three photo-cross-linking analogues that have been transcriptionally incorporated in vitro into RNA and used for RNA–protein cross-linking at internal uridine residues are 4-thio-UTP (Cramer et al., 1971; Bartholomew et al., 1987; Tanner et al., 1988), 5-bromo-UTP (Tanner et al., 1988), and 5-azido-UTP (Evans & Haley, 1987; Woody et al., 1988). All of these analogues have their cross-linking groups attached directly to the uridine base, allowing only close molecular contacts with the RNA to be examined. None of these analogues contains cleavable bonds.

We describe here the synthesis of a new UTP analogue that contains an aryl azide approximately 10 Å from the uridine which can be rapidly photoactivated by irradiation with long-wavelength ultraviolet light (300 nm). The azide group makes this analogue useful not only for examining RNA–protein interactions but RNA–DNA and RNA–RNA interactions as well. In addition, this analogue contains a sulfur atom between the nucleotide and the cross-linking group which

† Supported by an American Cancer Society grant (NP-544) and an Irvine Faculty Research Award to M.M.H. and NIH Predoctoral Training Grant GM07134-14 to S.D.

* Correspondence should be addressed to this author.

0006-2960/89/0428-5814$01.50/0

can be removed by Raney nickel (Raney, 1933; Hauptmann & Walter, 1962; Bonner & Grimm, 1966), leading to bond scission and making reversal of bimolecular cross-links possible when desired.

Experimental Procedures

Materials

All reagents and solvents were of reagent grade and used without further purification unless noted otherwise. Deionized and distilled water was used throughout. Water used for final reconstitution of nucleotides and all reagents used for transcription reactions and cross-linking were treated with 0.1% DEPC[1] before use to inactivate ribonucleases. PEI–cellulose F TLC plates were from VWR. *E. coli* MRE 600 cells were from Grain Processing Corp.

Buffers and Solvents. The buffers used were as follows: buffer A, 0.02 M TEAB, pH 8.3; buffer B, 2 M TEAB, pH 8.3; buffer C, 0.11 M Tris-HCl (pH 8.9) and 0.11 M NaCl; buffer D, 50 mM Tris-HCl, pH 8; buffer E, 20 mM Tris–OAc (pH 7.5), 10 mM $Mg(OAc)_2$, 50 mM potassium glutamate, 5% (v/v) glycerol, 40 μM Na_2EDTA, and 40 μg/mL acetylated bovine serum albumin; buffer F, 20 mM Tris–OAc (pH 7.5), 5% (v/v) glycerol, 1 mM $Mg(OAc)_2$, 0.1 mM Na_2EDTA, and 50 mM potassium glutamate; buffer G, 1.5 M NH_4OAc, 37.5 mM Na_2EDTA, and 50 μg/mL tRNA; buffer H, 0.3 M NaOAc and 10 mM $MgCl_2$; buffer I, 80% (v/v) formamide, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol, and 10 mM Na_2EDTA; buffer J, 0.05 M Tris–borate (pH 8.3) and 1 mM EDTA. The solvents used were as follows: solvent A, 1 M ammonium formate, pH 4.6; solvent B, 0.2 M LiCl; solvent C, 1.0 M LiCl; solvent D, 1.6 M LiCl.

Reagents. Azidophenacyl bromide was from Pierce. Raney nickel (W-2), dimethylacetamide, sodium borohydride, sodium sulfide nonahydrate, sulfur (99.99%, Gold Label), and bromine were from Aldrich. DE52 resin was from Whatman Biosystems Ltd.

Nucleotides. FPLC pure ribonucleoside triphosphates, 3′-deoxy-ATP, and 3′-*O*-methyl ribonucleotides used for transcription were from Pharmacia. Adenylyl(3′-5′)uridine (ApU), nucleosides, and nucleotides used for analogue synthesis and characterization were from Sigma.

Enzymes. Calf intestine alkaline phosphatase and snake venom phosphodiesterase were from Boehringer-Mannheim. Restriction endonucleases *Cla*I and *Sal*I were from New England Biolabs. *E. coli* RNA polymerase was isolated from *E. coli* MRE 600 cells by the method of Burgess and Jendrisak (1975) with the modifications of Lowe et al. (1979).

DNA Template. Plasmid pAR1707 (Studier & Rosenberg, 1981) DNA was isolated by the alkaline lysis method of Birnboim and Doly (1979) and purified by two successive CsCl gradient centrifugations. The T7 A1 promoter-containing fragment was prepared by digestion of pAR1707 with restriction endonucleases *Sal*I and *Cla*I. Enzymes were removed by phenol extraction, DNA was precipitated with ethanol and resuspended in water, and the 3′ recessive ends were filled in with Klenow fragment as described by Maniatis et al. (1982). The vector DNA was removed from the restriction fragment by precipitation with 5% poly(ethylene glycol) (Lis, 1980; Lis & Schlief, 1975). The restriction fragment was precipitated from the supernatant fraction with ethanol and resuspended in DEPC-treated water for transcription.

Methods

HPLC. Both preparative and analytical HPLC separations were carried out on a Vydac 300 ICB HPLC column (10 × 250 mm, 15–20 μm resin). For preparative HPLC, products were eluted with the following gradient of solvent A: 30–35% in 20 min; 35–44% by 110 min; 44–100% by 120 min. Solvent flow rate was 2.0 mL/min, and product elution was monitored at 280 nm. For analytical HPLC, a linear gradient of 0–100% solvent A in 30 min (3.5 mL/min) was used, and product elution was monitored at 254 nm.

Synthesis of 5,5″-Dithiobis(uridine 5′-triphosphate). Bis-5-SUTP (Figure 1) was synthesized by using a modification of the synthetic procedure for the nucleoside (Bardos & Kalman, 1966) and the nucleoside 5′-monophosphate (Ho et al., 1976). Synthesis is described for 200 μmol of bis-5-SUTP. Extremely detailed experimental procedures for its synthesis are described elsewhere (Hanna, 1988) and should be followed very carefully. Briefly, UTP (0.2 g, 400 μmol) was dissolved in 12.5 mL of dimethylacetamide at 4 °C. Methyl hypobromite (Duschinsky et al., 1967) was prepared in 7.5 mL of anhydrous methanol from 2.8 g (10 mmol) of silver carbonate and 0.55 mL (10 mmol) of bromine. Sodium disulfide was prepared from 2.3 g (9.6 mmol) of sodium sulfide nonahydrate and 0.31 g (9.6 mmol) of sulfur in 25 mL of 100% boiling ethanol. The methyl hypobromite was added to the suspension of UTP–dimethylacetamide, followed by addition of 2.3 g (8 mmol) of freshly prepared sodium disulfide.

Products were separated on a DE52 column equilibrated in buffer A and eluted with a linear gradient from buffer A to buffer B, as described (Hanna, 1988). Absorbance of eluting fractions was monitored at 273 and 332 nm before and after the addition of DTT to 2 mM. The disulfide-containing bis-5-SUTP absorbs at 273 nm. When the disulfide bond is cleaved with DTT, the 5-SH-UTP formed has an absorption maximum at 332 nm at pH 8 (Bardos & Kalman, 1966). Fractions that had absorption maxima at 273 nm were pooled, and the solvent was removed by lyophilization. In these experiments, the product was lyophilized for 3–5 days with several reconstitutions with water to remove salt. The yield of bis-5-SUTP was approximately 50% based on UTP and can be estimated by adjusting the solution to pH 8, reducing the disulfide bond with DTT, and using the absorbance of the resulting 5-SH-uridine nucleotide ($E_{332} = 8.82 \times 10^3$ M^{-1} cm^{-1} for 5-SH-UMP) (Ho et al., 1976).

Synthesis of 5-Mercaptouridine 5′-Triphosphate. Synthesis is described for 20 μmol of product, starting from 0.5 mL of 20 mM bis-5-SUTP. All remaining reactions were done on ice. 5-SH-UTP was prepared by the reduction of bis-5-SUTP (20 mM) with sodium borohydride in 0.5 mL of water (adjusted to pH 9.0 with concentrated ammonium hydroxide). Sodium borohydride (0.4 mg/μmol of bis-5-SUTP) was added, and the solution was mixed gently and left for at least 2 h with the cap open. The borohydride reduction reaction was quenched by the gradual addition of glacial acetic acid (approximately 10 μL at a time) until the pH was 4–5 and no more hydrogen gas was evolved. The pH was adjusted to 9.0 with ammonium hydroxide, and the 5-SH-UTP concentration was determined by measuring absorbance at 332 nm.

Synthesis of 5-[(4-Azidophenacyl)thio]uridine 5′-Triphosphate. All remaining manipulations were carried out in the dark or under red light. 5-APAS-UTP was prepared

[1] Abbreviations: APB, *p*-azidophenacyl bromide; 5-APAS-UTP, 5-[(4-azidophenacyl)thio]uridine 5′-triphosphate; bis-5-SUTP, 5,5″-dithiobis(uridine 5′-triphosphate); CIP, calf intestine alkaline phosphatase; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; SVP, snake venom phosphodiesterase; TEAB, triethylammonium bicarbonate; TLC, thin-layer chromatography; Tris,tris(hydroxymethyl)-aminomethane; UTP, uridine 5′-triphosphate.

(Figure 2) by alkylation of 5-SH-UTP with azidophenacyl bromide (APB). 5-SH-UTP (20 μmol) was prepared as described; however, the reduction reaction was not quenched until the APB had been dissolved. APB (80 μmol) was dissolved in 3 mL of 85% (v/v) dimethyl sulfoxide by stirring rapidly at room temperature. The 5-SH-UTP was added immediately after quenching to the APB solution. The tube containing the 5-SH-UTP was rinsed with 1 mL of water, and this was added to the APB solution. The pH of the reaction mixture was adjusted to 9.0 with ammonium hydroxide or acetic acid, if needed, and the reaction mixture was stirred in the dark overnight at 4 °C.

The reaction was diluted to 20 mL with water and extracted three times each with 25 mL of water-saturated diethyl ether. The aqueous phase was taken to dryness by lyophilization, reconstituted with 5 mL of water, filtered through a 0.45-μm filter, and isolated by preparative HPLC. Column fractions were further analyzed by scanning UV spectroscopy, and the fractions that had absorption maxima at 300 nm were taken to dryness by lyophilization and reconstituted in DEPC-treated water for analysis. Concentrations were estimated by using the extinction coefficient for APB of $E_{300} = 2 \times 10^4$ M^{-1} cm^{-1} (Hixson & Hixson, 1975; Erecinska, 1977).

Enzyme Digestion Reactions. The two products eluting during preparative HPLC with absorption maxima at 300 nm that did not correspond to reactants were further characterized by enzymatic digestion with snake venom phosphodiesterase (SVP) or calf intestine alkaline phosphatase (CIP). These were designated product A (eluting at 42 min) and product B (eluting at 72 min). The SVP digestion reactions contained 2 μL of 4 mM nucleotide in water and 2 μL of 2 mg/mL enzyme (1.5 units/mg) in buffer C. CIP digestion reactions contained 2 μL of 4 mM nucleotide in water and 2 μL of enzyme (1 unit/mL) in buffer D. Enzyme digestions were done at 37 °C for 1 h in the dark.

Thin-Layer Chromatography. Products of the enzyme digestion reactions were analyzed on PEI–cellulose F TLC plates. Four-microliter sample aliquots were applied 2 cm above the bottom of the TLC plate. The TLC plates were developed in the dark with the following solvent gradient: solvent B for 4 min, solvent C for 12 min, and solvent D until the solvent front had moved 19 cm up the plate. TLC plates were not allowed to dry between solvent changes. Aromatic compounds were visualized by fluorescence quenching by irradiating the TLC plate with 254-nm light. Photosensitive compounds were located by irradiating the TLC plate with the 302-nm light for 10 min (Figure 3).

Raney Nickel Desulfuration and DTT Reduction Reactions. UTP, bis-5-SUTP, and 5-APAS-UTP reaction products A and B were treated with Raney nickel (20 g/g of nucleotide) at 37 °C in 0.2 M NaOAc, pH 5.0, for 60 min. Product adsorption onto the Raney nickel was reversed by addition of 6 μL of 1 M DTT and shaking at room temperature for 10 min. The Raney nickel was pelleted by centrifugation at 10000*g* for 5 min at room temperature, and the supernatant was analyzed by analytical HPLC. Bis-5-SUTP was reduced to 5-SH-UTP by incubation with 6 mM DTT in 10 mM Tris-HCl, pH 8, for 30 min at room temperature. Reduction products were analyzed by analytical HPLC.

Photolysis. Samples (0.2 mL, 0.1 mM) were irradiated in borosilicate tubes at room temperature for the indicated times 2 cm from a 302-nm medium-wavelength mercury vapor lamp (Spectroline Model XX-15B, 1800 μW/cm² at 15 cm). Absorbance from 250 to 350 nm was measured as a function of irradiation time. Spectra were overlayed, and the change in absorption due to the azide was measured (Figure 4).

Nuclear Magnetic Resonance Measurements. Approximately 1 mg of 5-APAS-UTP product B was reconstituted three times in 0.5 mL of 99.96% D_2O with intermittent lyophilization to dryness at room temperature in the dark. For proton and ^{31}P NMR, the sample was reconstituted in 0.5 mL of D_2O and placed in a 535PP NMR tube (5 mm, thin wall). For ^{13}C NMR, the sample was lyophilized to dryness, resuspended in 0.22 mL of D_2O, and placed in Varian matched susceptibility plugs placed directly in the NMR tube to increase resolution of the signal. The spectra were taken on a GN 500-MHz NMR spectrometer. The ^{13}C spectrum was taken overnight.

Atomic Distance Measurements. The distance between the nitrogen atom of the azide group that participates in the insertion reaction and other positions on the nucleoside were calculated by using the PC MODEL computer program (PC version of Clark Still's MODEL) (Figure 6). Several potential conformations involving rotation around bonds in the cross-linker arm were predicted by this program.

Preparation of A-20 Ternary Transcription Complexes. Transcription complexes on the T7 A1 promoter containing RNA 20 nucleotides long (A-20s) were formed at 30 °C. RNA polymerase–promoter complexes were formed at promoter A1 by incubating the following in 150 μL of buffer E for 1 min: 18 nM pAR 1707/*Cla*I/*Sal*I DNA fragment, 18 nM *E. coli* RNA polymerase holoenzyme, and 100 μM ApU. Transcription was initiated by addition of FPLC purified ATP, CTP, and GTP to 1 μM ([α-^{32}P]GTP = 4×10^6 cpm/pmol), and the reaction was incubated at 30 °C for 3 min to allow formation of the A-20 ternary transcription complex. Complexes were then separated from unincorporated nucleotides by chromatography on a 15 × 1 cm Sepharose 6B column equilibrated in buffer F. Fractions (400 μL) were collected, and 2-μL aliquots were spotted on GF/C filters and counted. Fraction numbers 10 and 11 contained the majority of the transcription complexes and were used to test transcriptional incorporation of the 5-APAS-UTP reaction products.

Incorporation of UTP Analogues into RNA with E. coli RNA Polymerase. All reactions were done in reduced light. The isolated transcription complexes were adjusted to 10 mM $Mg(OAc)_2$, warmed to 30 °C, and split into 50-μL aliquots. Transcription complexes containing RNA 21 nucleotides long were prepared by addition of UTP (to 10 μM) or UTP analogue (to 200 μM) to these A-20 complexes and incubation at 30 °C for 10 min. Transcription complexes containing RNA 22 nucleotides long were prepared by adding UTP (10 μM) and 3′-deoxy-ATP (200 μM) or UTP analogue (200 μM) and ATP (200 μM) to these complexes and incubating at 30 °C for 10 min. Transcription was stopped by addition of 2 volumes of buffer G, followed by precipitation of RNA with 3 volumes of ethanol. The RNA was resuspended in 100 μL of DEPC-treated water and reprecipitated with 3 volumes of 1 M NH_4OAc in ethanol. Finally, the RNA was resuspended in buffer I for analysis on a 40 × 0.075 cm 20% polyacrylamide–7 M urea gel in buffer J (acrylamide/methylenebis(acrylamide) = 19/1). The RNA was analyzed by electrophoresis in the dark at 1100 V until the bromophenol blue marker dye had migrated to the bottom of the gel. Autoradiography of the gel was at −80 °C with Kodak X-Omat AR-5 X-ray film and a Cronex Lightning Plus intensifying screen.

Synthesis of RNA Markers. RNA ladders were synthesized from the T7 A1 promoter by incorporation of base-specific RNA chain terminators. Transcription was initiated by incubation of 8 nM pAR1707/*Cla*I/*Sal*I DNA fragment, 80

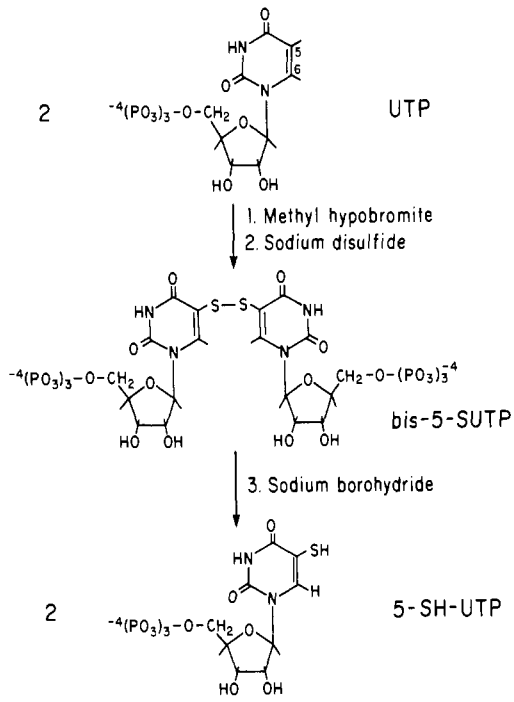

FIGURE 1: Synthetic scheme for bis-5-SUTP and 5-SH-UTP from UTP.

nM *E. coli* RNA polymerase, and 100 μM ApU in 60 μL of buffer E for 5 min at 37 °C. This was split into three 20-μL aliquots, and to each were added simultaneously one RNA chain terminator and ribonucleoside triphosphates to 1 μM ATP, 5 μM [α-^{32}P]CTP (5×10^4 cpm/pmol), 5 μM GTP, and 1 μM UTP. Terminating nucleotide concentrations were 100 μM 3′-*O*-methyl-ATP, 500 μM 3′-*O*-methyl-CTP, or 250 μM 3′-*O*-methyl-GTP. Reactions were left at 37 °C for 5 min, transcription was stopped by addition of 50 μL of buffer G, and RNA was precipitated with 3 volumes of ethanol. RNA was resuspended in 100 μL of buffer H, reprecipitated with ethanol, and resuspended in buffer I for gel electrophoresis.

RESULTS

Synthesis of Bis-5-SUTP and 5-SH-UTP. The activation and coupling of UTP to produce the dinucleotide disulfide (Figure 1) give a mixture of products. In addition to the coupled dinucleoside hexaphosphate, the presence of both UDP and UMP in the UTP leads to the formation of a number of mixed nucleotides ranging down to the dinucleoside diphosphate. Many of these products have the same charge and are very difficult to separate by anion-exchange chromatography. We have analyzed the DE52 product by analytical HPLC and see a major product eluting at 28 min and several minor components eluting earlier. The appearance of at least four compounds in the purified bis-5-SUTP when analyzed by TLC (see below) is consistent with the HPLC elution profile. The disulfide-containing compounds can be identified by their reaction with DTT to give 5-SH-nucleotides that do not absorb 254-nm light at pH 4.6 and are therefore not detected in this HPLC system. Desulfuration of the major bis-5-SUTP product yields exclusively UTP (eluting at 18 min). UTP treated with Raney nickel elutes with the same retention time as untreated UTP.

5-APAS-UTP Synthesis and Isolation. The alkylation of 5-SH-UTP with APB (Figure 2) gives one major (B) and one minor (A) product during purification by preparative HPLC. Product A elutes at 42 min, and product B elutes at 72 min. Both products have absorption maxima near 300 nm due to the aryl azide. Yields based on the amount of 5-SH-UTP used

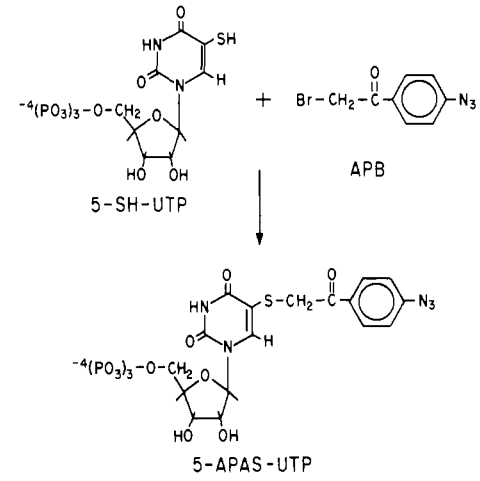

FIGURE 2: Synthetic scheme for 5-APAS-UTP from 5-SH-UTP and APB.

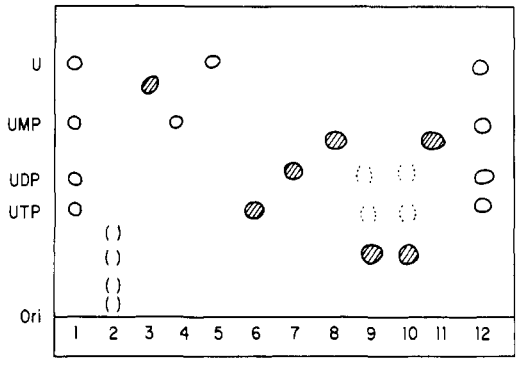

FIGURE 3: TLC analysis of 5-APAS-UTP reaction products and enzyme digestion studies. Samples were analyzed on PEI–cellulose F plates. Ori indicates the position at which samples were spotted. Lanes 1 and 12 contain uridine, UMP, UDP, and UTP controls. Lanes 2–11 contain the following: (lane 2) DE52 column purified bis-5-SUTP; (lane 3) APB; (lane 4) UTP + SVP; (lane 5) UTP + CIP; (lane 6) product A; (lane 7) product A + SVP; (lane 8) product A + CIP; (lane 9) product B; (lane 10) product B + SVP; (lane 11) product B + CIP. Spots that turned brown when the TLC plate was irradiated at 302 nm (indicating the presence of the azide group) are shaded.

and calculated by using the extinction coefficient for the aryl azide are approximately 18% for product A and 48% for product B. Product B corresponds to 5-APAS-UTP, which has a molecular weight of 675 as the free acid. This corresponds to a yield of 6.5 mg of the desired product.

Enzyme Digestion Studies. Products A and B were analyzed by TLC before and after digestion with enzymes specific for the hydrolysis of 5′-nucleotides to nucleosides (CIP) or cleavage of phosphodiester bonds to give 5′-monophosphates (SVP) (Figure 3). Control reactions with UTP show the conversion of UTP to UMP with SVP (lane 4) and the conversion of UTP to uridine with CIP (lane 5). For a given base, the greater the number of 5′-phosphate groups, the lower the R_f value in this TLC system. Products A and B had different R_f values before enzymatic digestion (A = 0.33, B = 0.21), with product B (lane 9) showing two minor contaminants (R_f = 0.33 and 0.46). One of these has the same R_f value as product A (lane 6). Treatment of both products with CIP gave an azide-containing nucleoside with an R_f value of 0.57 (lanes 8 and 11). Product A reacts completely to give a product containing fewer phosphates (lane 7, R_f = 0.46), while product B does not react at all with SVP (lane 10). Further, the minor product in product B that has the same R_f value as product A (0.33) also fails to react with SVP. Both the major (B) and minor (A) APAS-UTP reaction products turned brown on the

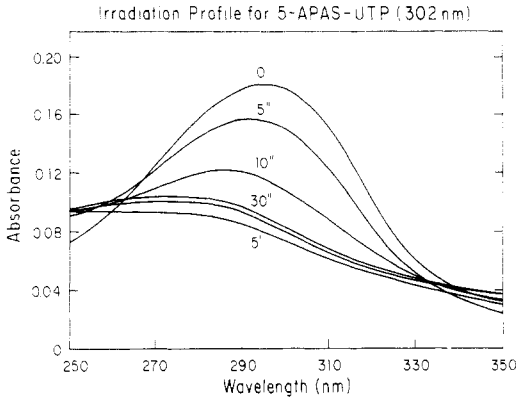

FIGURE 4: Irradiation profile of 5-APAS-UTP. Samples were irradiated with a 302-nm light source for the indicated times (0, 5, 10, 30, 60, 300 s), and the absorption spectra were overlayed. Absorption at 293 nm is due to the azide group, and a decrease in this absorbance indicates photoexcitation of the azide. Excitation is complete in approximately 15 s.

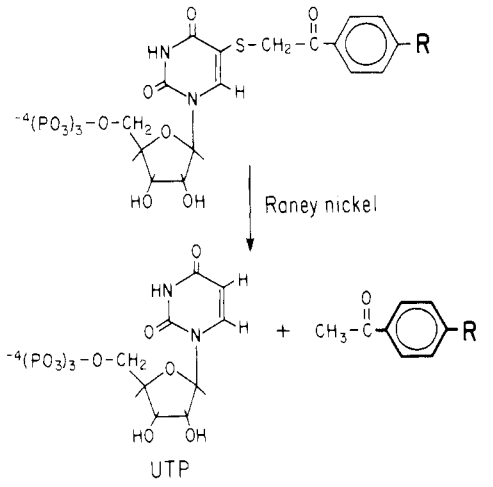

FIGURE 5: Raney nickel desulfuration of 5-APAS-UTP or a cross-linked product. Desulfuration of 5-APAS-UTP (R = N_3) results in the release of UTP and *p*-azidoacetophenone. If the analogue is cross-linked to another molecule (R = protein or nucleic acid), UTP is still released, along with a macromolecule now tagged with the acetophenone group.

TLC plate when irradiated with the ultraviolet light at 302 nm, indicating the presence of the aryl azide group. Irradiation of the TLC plate after application of the sample but before development of the plate resulted in retention of all ultraviolet-light-absorbing material at the plate origin (not shown). This is consistent with the presence of the azide group causing covalent attachment of the nucleotide to the TLC plate during irradiation.

Irradiation Studies. The azide groups of both products A and B have absorption maxima near 300 nm at pH 7.0, with half-lives of less than 10 s under our irradiation conditions. The absorption maximum for 5-APAS-UTP (product B) is at 293 nm, and the irradiation/absorption profile is shown in Figure 4. The profile for product A is only slightly different (not shown).

Raney Nickel Desulfuration. Products A and B were further characterized by desulfuration with Raney nickel. The desired product, 5-APAS-UTP, should give UTP when treated with Raney nickel (Figure 5). We found that desulfuration of product B did yield exclusively UTP, whereas desulfuration of product A gave only UDP (not shown). The Raney nickel cleavage to UDP indicates that product A is not the desired 5-APAS-UTP, and we have therefore further characterized only product B.

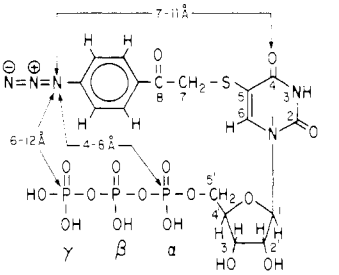

FIGURE 6: Distances between the azide and other atoms on 5-APAS-UTP. The nucleotide is drawn in the conformation that would be necessary for hydrogen bonding to other nucleotides through base pairing. This is the conformation that would be required for incorporation of the analogue into an RNA chain with RNA polymerase. Distances between the cross-linking nitrogen atom in the azide and the base C-4 or the α or γ phosphates were calculated with the PC MODEL computer program after structural energy minimization. Several conformations were analyzed allowing rotation around the single bonds in the cross-linking arm, and this is reflected in the range of distances shown.

NMR Analysis. The structure of product B was further analyzed by ^1H, ^{13}C, and ^{31}P NMR (not shown). See Figure 6 for numbering system of 5-APAS-UTP. The proton NMR shows an aryl AA'BB' doublet at 7.2 and 7.9 ppm, C-6 H at 8.1 ppm, C-7 H at 4.2 ppm, C-4' H at 5.9 ppm, and the N-3 H just visible at 4.3 ppm. The carbon-13 NMR was necessary to definitively define the structure with respect to pyrimidine ring substitution. The following resonance assignments were made from values listed in Jones et al. (1970): aryl carbons, 119 and 131 ppm; aryl C-8 carbonyl, 171 ppm; C-6 carbon, 146 ppm. The presence of all three 5' phosphates was verified with the ^{31}P NMR, with the following assignments: α phosphate, −14 ppm; β phosphate, −23 ppm; γ phosphate, −8 ppm. These results are consistent with the results of Roby et al. (1987) for the phosphates of UTP.

Transcription Studies. All of the products from the preparative HPLC separation, both major and minor, were assayed for their ability to act as a substrate for transcription with *E. coli* RNA polymerase. We found that only the two major products could be incorporated and that both could be placed at internal positions within the RNA chain (Figure 7). Mobilities for analogue-containing RNA are somewhat slower than the corresponding UTP-containing RNA.

Discussion

We report here the synthesis and characterization of a new photo-cross-linking UTP analogue. We have isolated this nucleotide analogue by HPLC and identified it as 5-[(4-azidophenacyl(thio)]uridine 5'-triphosphate (5-APAS-UTP). The structure was verified by enzyme digestion studies with CIP and SVP, NMR (^1H, ^{13}C, and ^{31}P), and Raney nickel desulfuration. The absorption maximum for the azide group is 293 nm, with the azide having a half-life of only seconds when irradiated with a 302-nm light source. We have shown that this nucleotide can be incorporated at terminal or internal positions within an RNA molecule by *E. coli* RNA polymerase.

This UTP analogue differs from the other photo-cross-linking UTP analogues that can be incorporated at internal RNA positions in two respects. First, the presence of the sulfur atom between the base and the cross-linking group makes this a reversible cross-linker via Raney nickel desulfuration. Second, 5-APAS-UTP differs in the position and/or type of cross-linking group present. 4-Thio-UTP undergoes photooxidation of the sulfur group to give electrophilic intermediates that react with nucleophiles, with loss of the sulfur atom (Pleiss

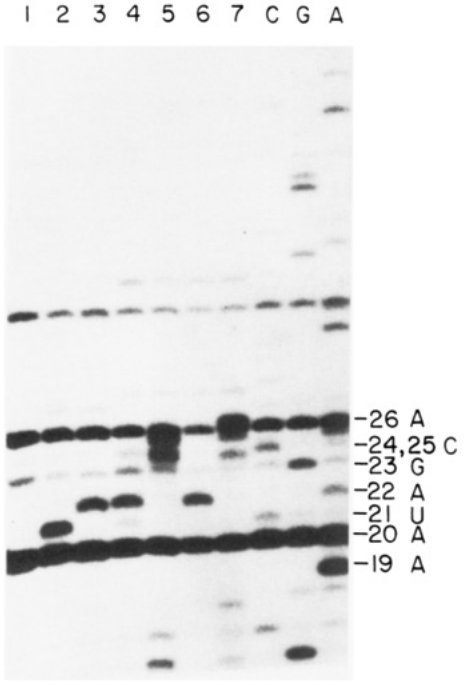

FIGURE 7: Incorporation of APAS-UTP products A and B into RNA with *E. coli* RNA polymerase. Transcription complexes (A-20s) were isolated as described. The only uridine residue in the first 20 nucleotides of the transcript is at position 2; therefore, transcription initiation with ApU results in RNA chains 20 nucleotides long to be formed by addition of only ATP, CTP, and GTP. The next four nucleotides in the RNA are UACG. The 20-mer RNA was elongated to 21 by the addition of UTP (or analogue) or to 22 by the addition of UTP (or analogue) and 3′-deoxy-ATP (or ATP). Lane 1 contains the column-isolated A-20s. Lanes 2–7 contain reactions in which the following were incubated for 10 min at 30 °C with the isolated A-20 complexes: 2-UTP; 3-UTP + 3′-deoxy-ATP; 4-APAS-UTP product A; 5-APAS-UTP product A + ATP; 6-APAS-UTP product B; 7-APAS-UTP product B + ATP. Marker lanes (C, G, A) contain RNA sequencing ladders. The size of the RNA and the base found in the 3′ end of the RNA is shown on the right. The analogue-containing RNAs have slower mobilities than UTP-containing RNA. Analogue-containing 21-mer migrates as a UTP-containing 22-mer. Analogue-containing 22-mer migrates as a UTP-containing 24-mer (product A) or 26-mer (product B). Contamination of the nucleotides with some CTP is responsible for the 25-mer seen in lane 5.

& Cerutti, 1971; Hajnsdorf et al., 1986). As the cross-linking functional group is generated through photoexcitation of the pyrimidine through the sulfur atom, 4-thiouridine can be used to examine contacts with RNA within only a few angstroms. This is true for 5-bromo-UTP as well. Cross-linking through this analogue occurs by the formation of a free radical as bromine is photolytically cleaved from the ring during photoexcitation. The bromine free radical is considerably more long-lived than the nitrene formed from an azide, and the radical can diffuse and cause nicking of nucleic acids at other locations (Monkehaus & Kohnlein, 1973). Cross-links formed with both of these analogues, as well as 5-azido-UTP, are to molecules in direct contact or only a few angstroms away from the base itself.

The nitrene formed upon photoexcitation of 5-APAS-UTP is at some distance from the base, ranging from 7 to 11 Å from the C-4 atom of the base for the orientation shown in Figure 6, depending on the conformation of the molecule. Using the PC MODEL program, several low-energy conformations for this molecule were generated by rotations around bonds in the cross-linking arm, assuming a fairly fixed conformation for the rest of the molecule. The anti conformation shown in Figure 6 is that which would be required for incorporation into RNA with *E. coli* RNA polymerase, since the functional groups at positions 3 and 4 of the base are involved in base pairing with the DNA template.

We believe this UTP analogue will be very useful for examining the interactions between internal nucleotides in an RNA chain and proteins or other nucleic acids. Since the 5-position of the uracil is not involved in normal Watson–Crick base pairing, RNA containing this analogue should form normal secondary and tertiary structures, providing there is no interference from the cross-linking arm. Complete substitution of 5-APAS-UTP for UTP in longer RNA still results in the correct termination of transcription at a ρ-independent termination site, suggesting that a normal stem–loop termination structure forms in the RNA (Dissinger and Hanna, unpublished results).

By synthesis of analogue-containing RNA (with a 5-APAS-UTP to UTP ratio that allows the formation of normal RNA secondary structures), interactions between these structures and adjacent molecules can be examined by photochemical cross-linking. Cross-linking effectively "traps" the molecular interaction covalently, thus providing a stable substrate for physical and biochemical analyses. We have carried out cross-linking experiments with this analogue to examine contacts between *E. coli* RNA polymerase and the 3′ and internal positions in nascent RNA during transcription (Dissinger and Hanna, unpublished results).

We believe the desulfuration of this nucleotide analogue will be possible even when the analogue is involved in a cross-link between RNA and proteins or nucleic acids. Such desulfuration will allow the separation of analogue-containing RNA from molecules to which it is cross-linked after isolation of the cross-linked complex. Such separation of molecules involved in a cross-linked complex greatly facilitates the identification of these molecules. Further, by using tritiated Raney nickel, it should be possible to leave a radioactive tag on whatever molecules have become cross-linked to the nucleotide when the complexes are separated by Raney nickel, as the hydrogens are provided by the catalyst (see Figure 5).

The structure of HPLC product A has not yet been determined. The Raney nickel desulfuration of product A yields only UDP, suggesting that this might be simply the diphosphate form of 5-APAS-UTP. Consistent with this is the appearance of breakdown products of 5-APAS-UTP over time that have the same R_f values as product A and the monophosphate of product A (Figure 3). Inconsistent with this assignment, however, is the fact that the 5-APAS-UTP breakdown product with the same R_f value as product A does not react with SVP, as does product A. Also inconsistent with product A simply being 5-APAS-UDP is the fact that it acts as a substrate for *E. coli* RNA polymerase (Figure 7). We find no incorporation of UDP into RNA under the same conditions used in this experiment. Whatever its structure, it also acts as a photoprobe of RNA polymerase (not shown), and we are working to determine its structure.

ACKNOWLEDGMENTS

We thank Craig Beeson and Laurel Hacche for help in execution and analysis of the NMRs, Matthew Thomas and Walter Tien for technical assistance, and Daniel Riggs for critical reading of the manuscript.

Registry No. 5-APAS-UTP, 120853-61-4; bis-5-SUTP, 120853-62-5; UTP, 63-39-8; 5-SH-UTP, 120853-63-6; 4-azidophenacyl bromide, 57018-46-9.

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