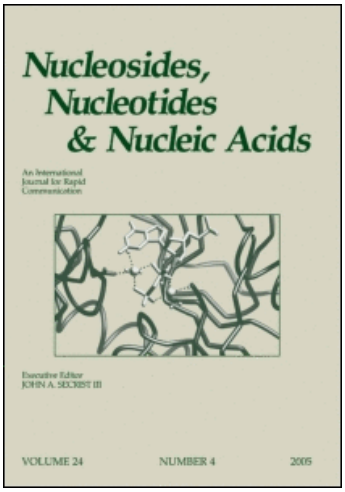

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A CONVENIENT PREPARATION OF A NEW C-5 BIOTINYLATED dUTP DERIVATIVE

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Abstract. An improved method for the C-5 biotinylation of dUTP is herein described. Palladium-catalyzed condensation of conjugated olefin 3 with mercurated dUTP 4 gave C-5 biotinylated dUTP derivative 5 in 50-70% yield. Enzymatic characterization of 5 demonstrated versatile substrate activity for incorporation into DNA.

The ability of pyrimidine nucleoside triphosphates carrying substituents at the C-5 position of the heterocyclic ring to act as substrates for various polymerases has been exploited as a vehicle for introducing nonisotopic labels into nucleic acid polymers. Langer *et al.* reported the synthesis of biotin-containing dUTP analogs and described methods for incorporating these nucleosides into DNA.[1] Subsequent papers[2] described the use of biotinylated DNA as hybridization probes. The availability of these compounds opened up a new horizon in DNA hybridization methodology.

The essential feature of the synthesis of Langer's biotinylated dUTP derivatives is the organopalladium-promoted coupling of C-5 mercurated dUTP with allylamine under mild conditions.[1] Although Langer did not publish the yield for this step, another group reported it at 20%.[3] In our attempts to condense C-5 mercurated dUTP (4) with allylamine, we found the reaction to be very inefficient and encountered purification difficulties. Because of these inherent problems, we designed an alternate route to synthesize a C-5 biotinylated analog in a more convenient and efficient way.

Our synthetic scheme, as outlined in Scheme I, allows for a conjugated olefin (3) to be coupled with C-5 mercurated dUTP 4. Since

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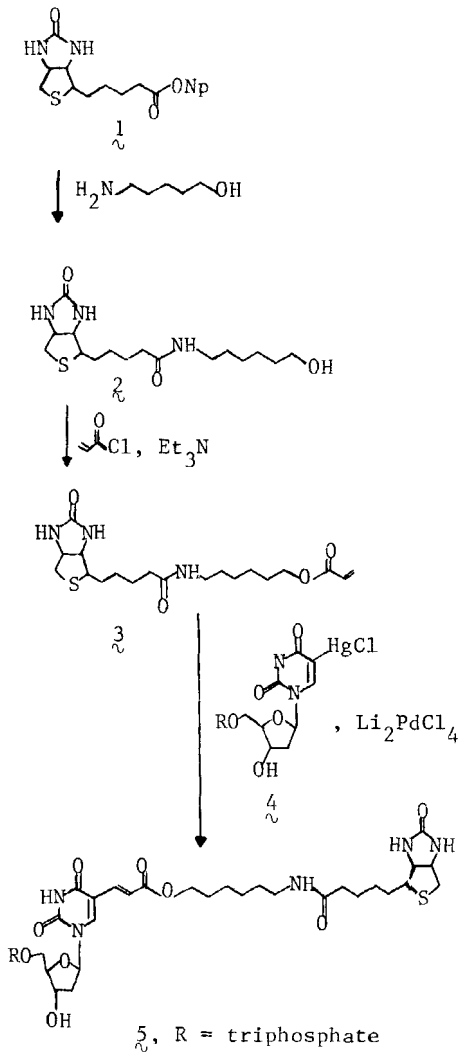


SCHEME I

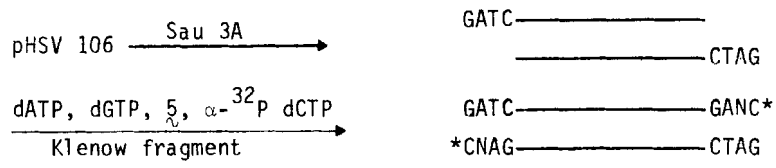
the palladium-catalyzed condensation of a conjugated olefin should facilitate the reaction and result in regioselective coupling between C-5 and the terminal alkenyl carbon to give a trans olefin[4], we envisaged a significant improvement in the yield of the reaction. Moreover, since this coupling is the final synthetic step, unnecessary nucleoside triphosphate purification would be eliminated.

Treatment of d-biotin-p-nitrophenyl ester (1) with 1 equivalent of 6-amino-1-hexanol in dry tetrahydrofuran precipitated 2 in 96% yield.

Biotin derivative 2 was reacted with 2.2 equivalents of acryloyl chloride to afford acrylate 3 which was isolated by column chromatography. Mercurated nucleotide 4[1,5] and conjugated olefin 3 were successfully coupled with lithium tetrachloropalladate in aqueous methanol to give biotinylated triphosphate 5. The organopalladium coupling reaction yield was determined to be 55% by UV absorption. Purification was easily accomplished by a combination of Sephadex DEAE A-25 and AGTHIOL (to remove unreacted 4) chromatographies. The ultraviolet spectrum (pH 7.5) of 5 showed two characteristic absorptions at 271 (ε = 9350) and 302 (ε = 11,100). The ^{1}H NMR spectrum demonstrated downfield doublets at 7.48 (J = 16 Hz) and 6.92 (J = 16 Hz) indicative of a trans olefin and a C-6 pyrimidine singlet at 8.19. Also, a FAB mass spectrum of 5 gave negative molecular ion peaks at 862 (M-H) and 884 (M^+Na-2H).

Biotinylated dUTP 5 was further characterized by testing its ability to act as a substrate for various enzyme reactions (Table I). Klenow fragment repair of Sau 3A-digested pHSV 106 DNA[6] clearly exhibited the ability of 5 to be incorporated. Sau 3A digest (↓ GATC) was selected so that the repair of the sticky ends necessitated incorporation of 5 before the designated radiolabel α-^{32}P dCTP (see Scheme II). Therefore, the measured counts after isolation by binding to avidin-labeled latex beads[7] demonstrated the incorporation of 5. Nick translation of the same DNA exhibited the substrate activity of dUTP derivative 5 in relation to DNA polymerase I. By employing again α-^{32}P dCTP and 5 in the triphosphate pool, the isolated (avidin-latex bead[7] binding) counts were an indication of the incorporation of 5. Controls for both polymerase reactions were conducted by substituting dTTP for 5 and are included in Table I. The background exhibited by the controls was attributed to nonspecific sticking of the DNA to the avidin-labeled latex beads. Finally, incorporation of 5 was observed in the 3'-end tailing of synthetic oligonucleotide ^{32}P-GCCCCAGAGCAACGCG with terminal deoxynucleotidyl transferase (Figure I). As shown in lane A of Figure I, marked tailing was demonstrated with 5.

Although a thorough enzymological study of 5 has not been performed, its versatile ability to incorporate into DNA appears promising. Moreover, the described preparation of 5 offers a more convenient and efficient approach to C-5 labeling of dUTP.



*radioactive label N = nucleotide corresponding to 5

SCHEME II

TABLE I

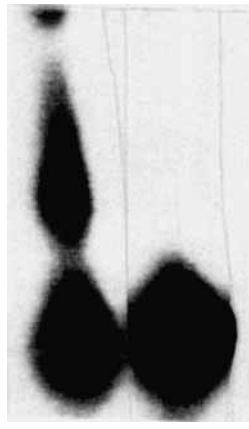
Incorporation of Biotinylated dUTP 5 as a Function of Incorporated α-^{32}P dCTP

	CPM
Klenow fragment repair of Sau 3A-digested pHSV 106 with 5	55,510
Klenow fragment repair of Sau 3A-digested pHSV 106 with dTTP (control)	4,555
Nick translation of pHSV 106 using 5	146,770
Nick translation of pHSV 106 using dTTP (control)	22,700

The isolation of biotinylated pHSV 106 DNA was accomplished by binding to avidin-labeled latex beads[7] (see experimental section).

EXPERIMENTAL

Lithium tetrachloropalladate was purchased from Aldrich Chemical Company. All enzymes, AGTHIOL (agarose-ethane-thiol), and dNTP's were obtained from Pharmacia P.L. Biochemicals. Radiolabeled nucleotides α-^{32}P dCTP and γ-^{32}P ATP were purchased from New England Nuclear. pHSV 106 plasmid DNA was purchased from Bethesda Research Laboratories. Oligodeoxynucleotide GCCCCAGAGCAACGCG was synthesized in collaboration with Dr. Chinh Bach (Syntex Corporation) and the 5'-terminal labeled with ^{32}P by published procedures.[12] The ultraviolet spectrum was obtained with a Hewlett-Packard 8450 UV/Vis Spectrometer. Proton magnetic resonance measurements were made with a Bruker WM-300 using either tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (aqueous solution) as internal standards. The mass spectrum (FAB) was recorded by Dr. D. H. Williams (Cambridge University, England) on a Kratos MS-50 instrument using



10% polyacrylamide gel: A) 3' tailing of 5'-^{32}P-GCCCCAGAGCAACGCG with 5; B) 5'-^{32}P-GCCCCAGAGCAACGCG (control).

FIGURE I

Terminal deoxynucleotidyl transferase tailing of ^{32}P-GCCCCAGAGCAACGCG

xenon as the primary beam and glycerol as solvent. Elemental analyses were determined by Syntex Analytical Research Division.

6-(N-biotinyl)-amino-1-hexanol (2)

To a solution of 6-amino-1-hexanol (165 mg, 1.41 mmol) in dry tetrahydrofuran (20 ml) was added d-biotin p-nitrophenyl ester (1, 500 mg, 1.37 mmol) and the mixture was stirred at room temperature. After 30 minutes, precipitation resulted in a thick slurry and more tetrahydrofuran (20 ml) was added. The mixture was stirred for 16 hours and the precipitate collected by filtration. Copious rinsing with tetrahydrofuran and diethyl ether afforded 453 mg (96%) of a pale yellow solid which melted at 190-191°. ^{1}H NMR (d_4-MeOH) δ 1.30-1.80 (m, 14H), 2.19 (t, J = 7 Hz, 2H, -CH_2CO-), 2.70 (d, J = 12.5 Hz, 1H, -CH_2S-), 2.92 (dd, J = 5, 12.5 Hz, 1H, -CH_2S-), 3.14-3.25 (m, 3H, contains -CH_2NH- triplet at 3.16, J = 7 Hz), 3.54 (t, J = 6.5 Hz, 1H, -CH_2OH), 4.29 (dd, J = 4.5, 7.7 Hz, 1H), 4.46 (dd, J = 4.5, 5 Hz, 1H); IR (KBr) 3300 br, 2945, 2873, 1681, 1643, 1545, 1482, 1272, 1069 cm^{-1}; EI mass spectrum m/e 301 (M+).

Anal. calcd. for $C_{16}H_{29}N_3O_3S$: C, 55.95; H, 8.51; N, 12.23. Found: C, 55.85; H, 8.53; N, 12.22.

6-(N-biotinyl)-amino-1-hexanol acrylate (3)

Compound 2 (405 mg, 1.18 mmol) was dissolved in dry dimethylformamide (10 ml) and cooled to 0° under a nitrogen atmosphere. Triethyl amine (360 µl, 2.6 mmol) and acryloyl chloride (210 µl, 2.6 mmol) were added, and the reaction mixture was warmed to ambient temperature. After stirring for 30 minutes, the solvent was evaporated *in vacuo* to a syrup and taken up in water (20 ml). The aqueous phase was extracted with methylene chloride (2 X 35 ml) and the extracts combined. After washing the organic phase with water (2 X 20 ml) and saturated brine (1 X 20 ml) and drying over anhydrous sodium sulfate, the solvent was removed *in vacuo* to give an oily residue. Chromatographic purification of the residue on a column of silica gel (2 X 55 cm) employing methanol-methylene chloride (1:9) elution afforded 106 mg (23%) of a white solid. mp 138-140°; ^{1}H NMR (d_4-MeOH) δ 1.32-1.80 (m, 14H), 2.19 (t, J = 7 Hz, 2H, $-CH_2CO-$), 2.70 (d, J = 12.5 Hz, 1H, $-CH_2S-$), 2.92 (dd, J = 5, 12.5 Hz, 1H, $-CH_2S-$), 3.14-3.25 (m, 3H contains $-CH_2NH-$ triplet at 3.17, J = 7 Hz), 4.15 (t, J = 6.5 Hz, 2H, $-CH_2OCO-$), 4.30 (dd, J = 4.5, 7.8 Hz, 1H), 4.47 (dd, J = 4.5, 5 Hz, 1H), 5.87 (dd, J = 1.7, 10.4 Hz, 1H, cis $=CH_2$), 6.15 (dd, J = 10.3, 17.3 Hz, 1H, $=CHCO-$), 6.39 (dd, J = 1.7, 17.3 Hz, 1H, trans $=CH_2$) IR (KBr) 3270 br, 2935, 2885, 1700, 1639, 1547, 1464, 1198, 1070 cm^{-1}. EI mass spectrum m/e 397 (M+).

Anal. Calcd. for $C_{19}H_{23}N_3O_4S$: C, 57.41; H, 7.86; N, 10.57. Found: C, 57.22; H, 8.12; N, 10.55.

Biotinylated dUTP (5)

Mercurated dUTP 4 (10 mg, 9 µmol) and acrylate 3 (50 mg, 126 µmol) were dissolved in methanol-water (9:1, 10 ml). A 20 mM methanolic solution of lithium tetrachloropalladate[14] (1 ml) was added, and the mixture was stirred for 17 hours at room temperature. Water (20 ml) was added, and the mixture was centrifuged. The liquid phase was added to a DEAE Sephadex A25 column (1 X 17 cm), washed with 0.05 M triethylammonium bicarbonate (TEAB), and eluted with a gradient of 0.05-1.0 M TEAB (600 ml total volume). Appropriate fractions that eluted at 0.5-0.6 M

TEAB were pooled (a mixture of 4 and 5) and evaporated *in vacuo* (30-35°) to dryness. After addition of water (10 ml) and reevaporation, the residue was dissolved in water (20 ml) and eluted through an AGTHIOL column (1 X 10 cm) using deionized water for rinsing. The total collected volume was concentrated, loaded on a small DEAE Sephadex A25 column (1 X 4 cm), and eluted with 0.6 M TEAB. The eluent was evaporated to dryness, and the remaining TEAB was removed by additional evaporation with water (10 ml). Dissolution of the residue in water (1 ml) and freeze drying afforded the desired triphosphate 5 as a light yellow solid. The yield was determined to be 55% by measuring UV absorbance at 302 nm (ε = 11,100). HPLC R_T = 5.71 min. [A = 40 mM ammonium phosphate (pH 7.0), B = 200 mM ammonium phosphate (pH 7.0), 5-30% B, 10 min.][8]; ^{1}H NMR (D_2O) δ 2.23 (t, J = 7 Hz, 2H, $-CH_2CO-$), 2.41 (m, 2H, 2'-H), 2.76 (d, J = 13 Hz, 1H, $-CH_2S-$), 2.97 (dd, J = 5, 13 Hz, 1H, $-CH_2S-$), 3.05 (m, 2H), 3.19 (t, J = 7 Hz, 2H, $-CH_2NH-$), 4.20 (m, 4H, contains $-CH_2CO-$ triplet at 4.21, J = 6.5), 4.39 (dd, J = 4.7, 8 Hz, 1H), 4.58 (dd, J = 4.7, 5.5 Hz, 1H) 6.30 (dd, J = 7 Hz, 1H, 1'-H), 6.92 (d, J = 16 Hz, 1H), 7.48 (d, J = 16 Hz, 1H), 8.19 (s, 1H); UV λ_{max} 271,302 nm (ε = 9350 and 11,100 respectively, pH 7.5).

Klenow fragment repair of Sau 3A-digested pHSV 106 DNA with 5

One µg of Sau 3A-digested pHSV 106 DNA[6] was treated with DNA polymerase I (Klenow fragment) in DNA polymerase buffer[9] containing 20 µM each of biotinylated dUTP 5 α-^{32}P dCTP, dGTP, and dATP. After incubation at 0° for 1 hour, the sample was heated for 1 minute at 70° to inactivate the enzyme. A parallel reaction containing dTTP instead of 5 was carried out as a control. To each of the polymerase reactions, 10^9 avidin-labeled latex beads[7] were added. After 10 min. incubation, the beads were centrifuged and the supernatant decanted. The beads were washed three times with Tris-saline buffer[13] and the radioactivity measured in a scintillation counter. Results are recorded in Table I.

Nick translation of pHSV 106 DNA using 5

The nick translation reactions were carried out using the conditions suggested by New England Nuclear (nick translation kit instruction manual). One µg of pHSV 106 DNA[6] was treated with Pancreas DNase I and DNA polymerase I in poly I nick translation buffer and

20 μM of dGTP, dATP, α-^{32}P dCTP, and 5. The reaction mixture was incubated at room temperature for 2 hours and the reaction terminated by heating at 70° for 2 minutes. A parallel reaction containing dTTP instead of 5 was carried out as a control. The reactions were worked up with avidin-labeled latex beads[7] as described in the Klenow fragment repair of Sau 3A-digested pHSV 106. Results are recorded in Table I.

Terminal deoxynucleotidyl transferase tailing of ^{32}P-labeled GCCCCAGAGCGCG

One pmol of ^{32}P-GCCCCAGAGCAACGCG was treated with 20 units of terminal deoxynucleotidyl transferase in terminal transferase buffer[11] containing 100 μM biotinylated deoxyuridine triphosphate. Reaction mixtures were incubated at 37° for 2 hours and then analyzed[13] on a 10% polyacrylamide gel. An autoradiogram is shown in Figure I.

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6. pHSV 106 DNA is a pBR 322 derivative containing a thymidine kinase gene sequence of Herpes Simplex Virus I inserted into the Bam HI restriction endonuclease site.

7. Latex particles were labeled with Avidin D by coupling with[8] EDAC. Biotin binding capacity was determined to be 70 pmol/6 X 10^8 beads.

8. A Varian Micropac NH_2-10 (4 mm X 30 cm) column was employed for this analysis.

9. 10 X DNA polymerase buffer: 0.5 M Tris (pH 7.2), 0.1 M $MgSO_4$, 1 mM DTT, and 500 μg/ml BSA.

10. Tris-saline buffer: 0.15 M NaCl, 50 mM Tris (pH 7.5), 10 mM EDTA, and 0.5% BSA.

11. 10X terminal transferase buffer: 1.4 M potassium cacodylate, 0.3 M Tris, 1 mM DTT, and 10 mM $CoCl_2$ (pH 6.9).

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14. Under similar reaction conditions, potassium tetrachloropalladate can be substituted for lithium tetrachloropalladate.

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