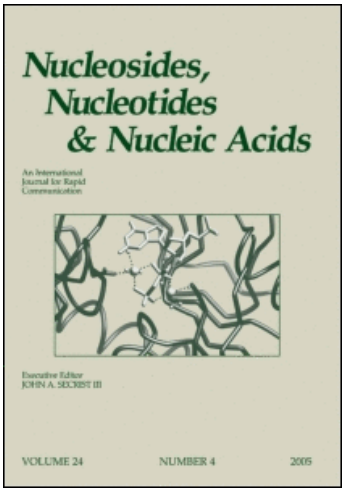


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# Synthesis of Radio-Labelled and Unlabelled $O^4$-Ethylthymidine 5'-Triphosphate

A. Bhattacharyya[a]; B. C. Pal[a]
[a] University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences and the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee, U.S.A.

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# SYNTHESIS OF RADIO-LABELLED AND UNLABELLED $O^4$-ETHYLTHYMIDINE 5'-TRIPHOSPHATE[1]

A. Bhattacharyya*,[2] and B.C. Pal

University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences and the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, U.S.A.



Abstract. This paper describes the synthesis of [$^3$H]-labelled and unlabelled $O^4$ethylthymidine 5'-triphosphate.

Introduction. In our previous communication[3] we reported the synthesis of unlabelled $O^4$-ethylthymidine 5'-triphosphate which could be used for studies on repair mechanism and other biological effects[4-13] of this modified base in a synthetic DNA polymer. Now we report the synthesis of radio-labelled $O^4$-ethylthymidine 5'-triphosphate of high specific activity, introducing the radioactivity by chemical reaction in the last step. The method has several advantages : (i) Radio-labelling will make the detection of small amounts of thymine moiety feasible for studies on repair mechanism. (ii) It will be possible to start the reaction from the first step on a large scale without problems of radioactive contamination throughout the reaction sequence. (iii) Also, such an approach will be more advantageous over the conventional method of using DNA treated with radio-labelled alkylating agents, since in that case the DNA contains multiple alkylated products and the yield of $O^4$-alkylthymine is rather small.

Materials. 5-Hydroxymethyl-2'-deoxyuridine, silica gel, bacterial alkaline phosphatase, 3'-nucleotidase were obtained from Sigma Chemical Company. All organic solvents were obtained from Fisher Scientific Company, and were distilled and dried before use. Ethylurea was



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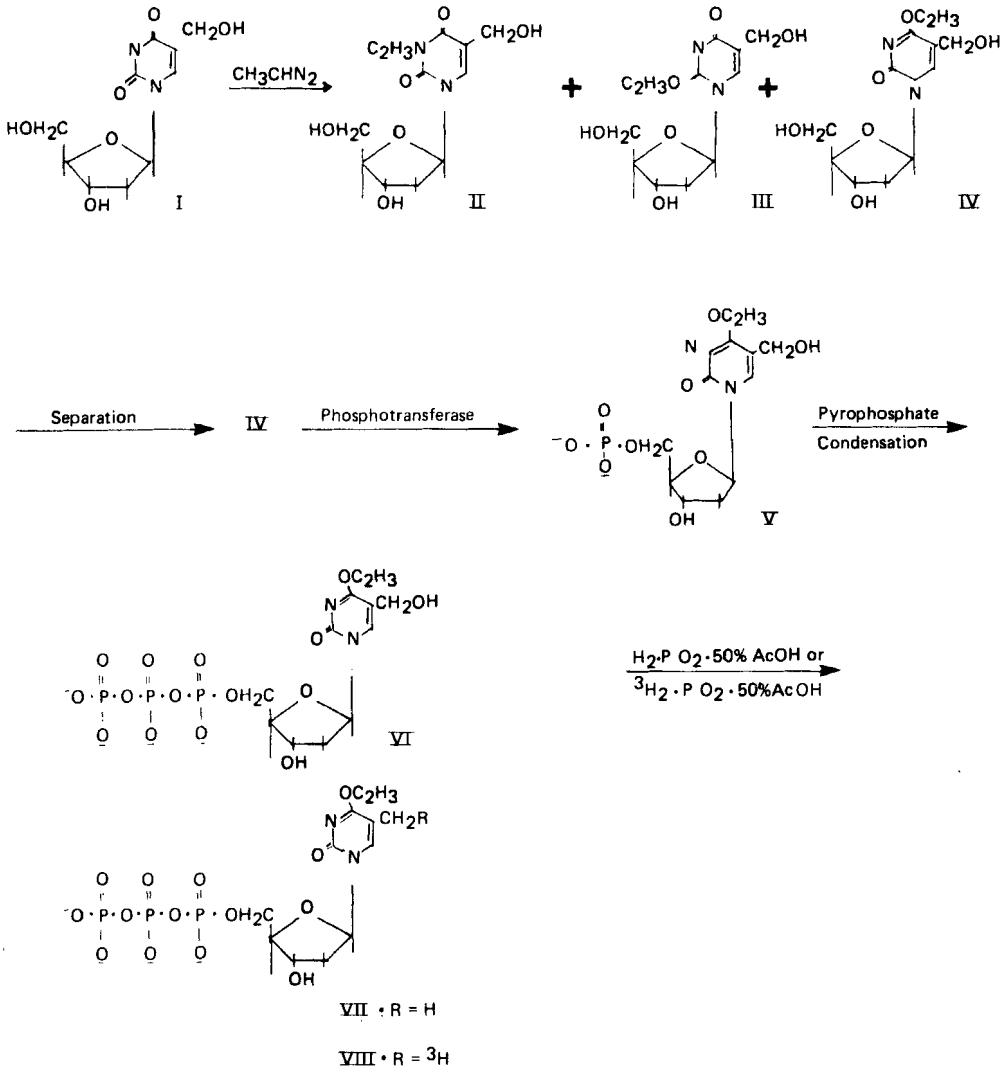
obtained from Aldrich Chemical Company for synthesis of ethylnitrosourea[14]. Diazoethane was prepared from ethylnitrosourea before use by the procedure of Arndt[15]. Triethylammonium bicarbonate was prepared by the procedure of Smith et al[16]. Carrot phosphotransferase was isolated according to the procedure of Strider et al[17] with the improvements suggested by Harvey et al[18]. Ultraviolet spectra were recorded on a Cary Model 14PM recording spectrophotometer, and nuclear magnetic resonance spectra were recorded on 60 MHz Varian T-60 spectrometer.

Synthesis and isolation of $O^4$-ethyl-5-hydroxymethyl-2'-deoxyuridine (IV). The solution of 5-hydroxymethyl-2'-deoxyuridine (I, 500 mg) in methanol (75 mL) was stirred in 250 mL round-bottomed flask at room temperature in hood, and an ethereal solution of diazoethane (0.07 M, 37 mL) was added all at once. After stirring the solution overnight at room temperature, the reaction mixture was examined by TLC on cellulose plate using n-butanol:ethanol:water (16:2:5 v/v/v), and the spots were observed under ultraviolet light. The starting material (I, $R_f$, 0.3) was absent, showing the conversion to be quantitative, and three new bands appeared indicating the formation of products N-3-(II), $O^2$-(III) and $O^4$-ethyl-5-hydroxymethyl-2'-deoxyuridine (IV). After evaporating the organic solvents in rotary evaporator under vacuum, the products were separated on a silica gel column (bed vol. = 150 mL) by step-wise elution with 5%, 10%, 15% and 20% methanol in chloroform. The appropriate fractions were pooled and characterized, after evaporation of solvents, by UV spectra, NMR and by studies on the stability of ether linkage and glycosyl bond[19]. The ethyl derivatives were eluted from the column in the order II, III, IV and the yields were 53.2%, 29.8% and 17.0% of theory respectively. All these compounds, II, III and IV, gave satisfactory elemental analysis for C, H, and N.

Synthesis, isolation and characterisation of V and VI were carried out according to the procedures described previously[3,20-24]. Yield of V, 65% of theory; $\lambda_{max}^{H_2O}$ = 275.5 nm; Phosphorus : nucleoside ratio = 0.9:1. Yield of VI, 20% of theory; $\lambda_{max}^{H_2O}$ = 276 nm; Phosphorus : nucleoside ratio = 3.08:1). Degradation of VI with potato apyrase and bacterial alkaline phosphatase produced IV, further confirming its structure[3].




Scheme 1

Synthesis and isolation of $O^4$-ethyl[Me-$^3$H]thymidine 5'-triphosphate (VIII). The compound VI (3.8 μmole) was dissolved in 140 μL of water, 140 μL of glacial acetic acid and 2.25 mg of platinum oxide catalyst[25] was added to the mixture stirred under $H_2$ (slightly above one atmosphere pressure) for 30 min at room temperature, to produce

$O^4$-ethylmidine 5'-triphosphate, VII, along with other products. Reduction of VI with $^3H_2$ at 660 mm Hg pressure under similar conditions produced $O^4$-ethyl[Me-$^3$H]thymidine 5'-triphosphate, VIII. The product VIII was isolated from the reaction mixture as follows : The reaction mixture was neutralized with calculated amount of $NH_4OH:H_2O$ = 1:1 (v/v) tc pH 7.0, and the exchanged tritium (in water and acetic acid) was removed by extensive washing with water and then with 0.1 M triethylammonium bicarbonate, pH 7.5 on a DEAE-Sephadex ($HCO_3^-$ form, bed vol. = 5 mL) column, and finally eluting the product VIII, along with other triphosphates, with 0.4 M triethylammonium bicarbonate, pH 7.5. The fractions containing the triphosphates were pooled and evaporated from 50% ethanol as before, and finally dissolved in 100 µL water, and injected into a C-18 µBondapak column (300 x 3.9 mm dia.), and eluted with 0.125 M ammonium phosphate, pH 7.0 at room temperature at a flow rate of 1 mL/min. The fractions containing VIII were pooled, and the ammonium phosphate was removed by DEAE-Sephadex column chromatography, eluting with 0.1 M triethylammonium bicarbonate, pH 7.5. The compound VIII was eluted with 0.4 M triethylammonium bicarbonate which was finally removed by evaporation as before, to give purified compound VIII. $\lambda_{max}^{H_2O}$ = 278 nm, yield = 22.3% of theory, specific activity = 1.5 Ci/mmole.



Results and Discussion. The ultraviolet spectral characteristics of the alkylated compounds II, III, IV are shown in Table 1.

Structures for II, III, and IV were assigned from spectral data and kinetic studies as follows : (i) Alkylation of pyrimidine nucleosides at N-3-, $O^2$- and $O^4$-positions affects the absorption maximum ($\lambda_{max}$) of the parent nucleoside in a definite pattern[19] : N-3-alkylation has very little effect on the position of $\lambda_{max}$ of the parent nucleoside, $O^2$-alkylation causes a blue shift of the $\lambda_{max}$ and the appearance of a new short wave length maximum at about 225 nm, whereas $O^4$-alkylation causes a red shift of the $\lambda_{max}$. Since the oxygen atom of the hydroxymethyl group in I is separated from the ring by a carbon atom, we assume that the oxygen atom will not interfere to any appreciable extent with the electronic level of the conjugated ring, and hence UV shifts due to alkylation should be similar to those of thymidine. Comparing the $\lambda_{max}$ values (Table 1) structures II, III, IV were

TABLE 1. Melting Points and Ultraviolet Spectral Characteristics of the Products Obtained by Alkylation of I. (Within brackets are the values of extinction coefficients in moles$^{-1}$ litre cm$^{-1}$ units.)

| Compound | m.p. °C | $\lambda_{max}$ (nm)* ($\varepsilon_{max}$) | $\lambda_{min}$ (nm)* ($\varepsilon_{min}$) |
|---|---|---|---|
| I | | 264(10187) | 232(2328) |
| II | 87-89 | 264(7690) | 235(2330) |
| III | 130-131 | 254(9280)<br>227(9385) | 239(7720)<br>215(>12682) |
| IV | 138-140 | 275(6090) | 230(1230) |

*Values at pH 7.0.



assigned to N-3-, $O^2$- and $O^4$-ethyl derivatives respectively. (ii) Of the alkylated pyrimidine nucleosides, N-3-alkyl derivative is most stable to acid hydrolysis, $O^2$-alkyl derivative is easily cleaved at the glycosyl linkage and $O^4$-alkyl derivative is easily cleaved at ether bond. The stabilities of the three ethylated derivatives are in agreement with the structures II, III, IV (Fig.1 and Fig.2).

All alkylated derivatives exhibit signals methyl H(β) and methylene H(α) protons and also lack the signal of N-H proton which is present in the nuclear magnetic resonance spectrum of I. As expected, the signals of methyl protons of N-3-ethyl derivative (triplet, ∿1.1δ ) are slightly upfield as compared to those of methyl protons of $O^2$- and $O^4$-ethyl derivatives (triplet, ∿1.3 δ ), and for methylene protons the difference is more prominent (Table 2). For $O^4$-ethyl derivative of uridines, there is a definite downfield shift of H(6) proton signal as compared to $O^2$-, N-3- or unalkylated compound[19]; this is also found in IV (Table 2). The H(α) proton signals are superimposed on those of 5-$CH_2$OH signals in $O^2$- and $O^4$-ethyl derivatives, and with H(3',4',5') in N-3-ethyl derivative (Table 2).

The elution profile for purification of VI is shown in Fig. 3.

To confirm the identity of VII (or VIII), $O^4$-ethylthymidine 5'-triphosphate was synthesized from thymidine by standard procedures[3]. The compound VII (or VIII) was found to be identical with $O^4$-ethyl-

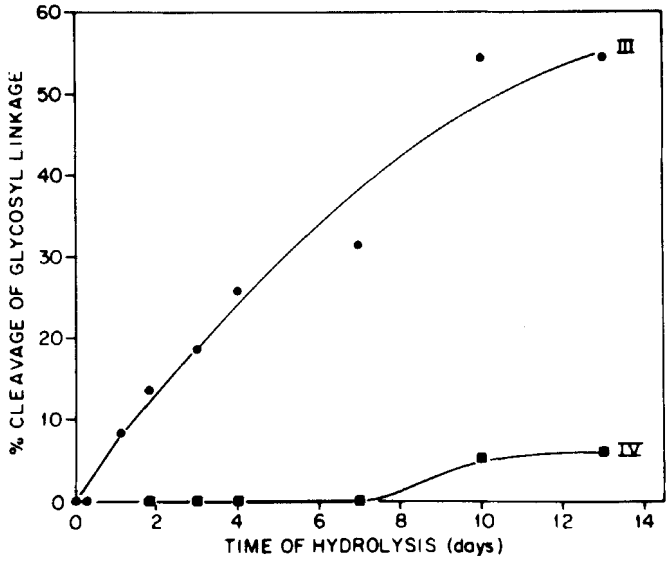



FIG. 1. (Left). Relative Stability of Glycosyl Bond. Compound II, III or IV (0.25 mg) is dissolved in 2.5 mL of 0.01N HCl, mixed well and allowed to stand at room temperature. This solution (100 µL) is injected into Aminex A-6 column (23 x 0.65 cm) at different time intervals, and eluted with 0.1 M ammonium bicarbonate, pH 7.5 at a flow rate of 0.31 mL/min at room temperature. 5-Hydroxymethyluracyl (retention time = 23.75 min) produced by cleavage of glycosyl bond, gives a measure of stability of the bond.



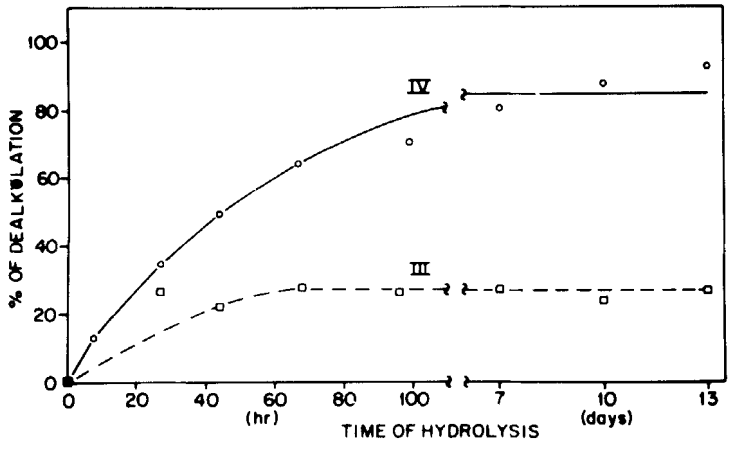



FIG. 2. (Right). Relative Stability of Ether Linkage. Hydrolysis and analysis by Aminex A-6 column (as before) is followed by the formation of I by cleavage of ether linkage of III or IV.

TABLE 2. Chemical Shifts ($\delta$ in ppm with respect to TMS as internal standard) for the Protons of I and its Ethyl Derivatives (in $d_6$-DMSO solution). (s = singlet, d = doublet, t = triplet, m = multiplet).

| Compound | H(β) | H(3',4',5') | H(α) | 5-$CH_2$ | H(N-3) | H(6) |
|---|---|---|---|---|---|---|
| I | - | 3.6-3.9 m | - | 4.21 broad | 11.5 broad | 7.98 S |
| II | 1.1 t | 3.64-4.0 m | | 4.28-4.32 d | - | 8.01 S |
| III | 1.31 t | 3.58-4.01 m | 4.26-4.67 m | | - | 7.98 S |
| IV | 1.28 t | 3.56-3.92 m | 4.25-4.58 m | | - | 8.29 S |

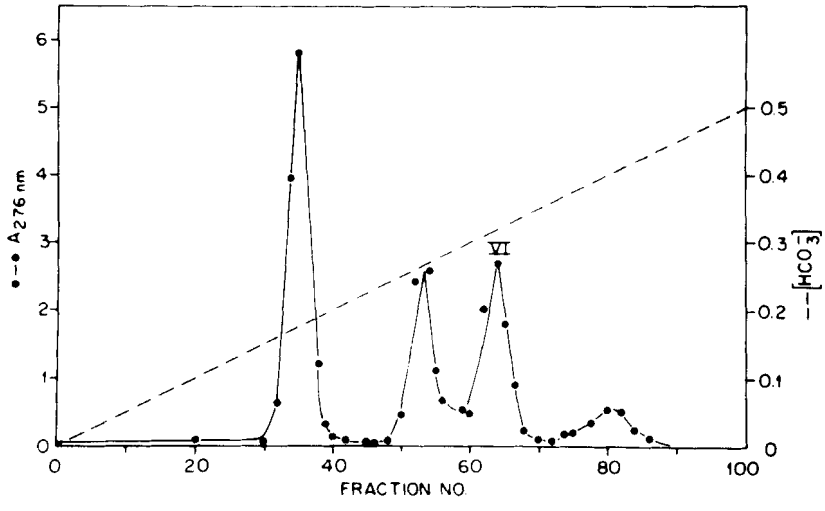



FIG. 3. Elution Profile for Purification of VI by DEAE-Sephadex Column. The triphosphate was identified by estimation of phosphorus. Details in the text.

thymidine 5'-triphosphate obtained from thymidine, with respect to (i) ultraviolet spectra (ii) retention times in HPLC (iii) $R_f$ values in TLC (iv) phosphorus:nucleoside ratio (v) degradation products obtained by the action of potato apyrase and bacterial alkaline phosphatase[3].

The present study affords a method for synthesis, purification and identification of radio-labelled $O^4$-ethylthymidine 5'-triphosphate. Incorporation of the triphosphate into DNA polymer and studies on the repair mechanism $O^4$-ethylthymine in synthetic DNA are underway in our laboratory.

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