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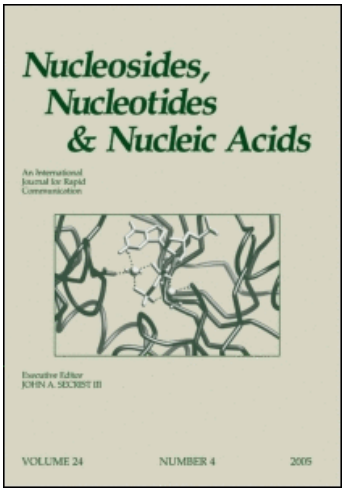

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SYNTHESIS OF O^2- AND O^4-ETHYLTHYMIDINE 5'-TRIPHOSPHATES[1]

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Abstract. This paper describes the microscale synthesis of O^2- and O^4-ethylthymidine 5'-triphosphates.

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Introduction. Simple alkylating mutagens and carcinogens can alkylate DNA at various nucleophilic sites, and any structural modifications of the genetic material may affect its replication as well as transcription[3,4]. Some of the alkylated pyrimidines, e.g., O^2- and O^4-alkylthymine, should directly affect the base-pairing behavior of these bases[5], and have been speculated as potentially mutagenic[6,7]. In the present study we describe the synthesis of O^2- and O^4-ethylthymidine 5'-triphosphates which may be used for studies on repair mechanisms and other biological effects of these alkylated bases in a synthetic DNA polymer. The choice of ethyl derivatives, in contrast to methyl derivatives, is based on the finding that O^4- and O^2-ethylation is more predominant compared with O^4- and O^2-methylation when poly[dA-dT] is treated with ethylnitrosourea and methylnitrosourea respectively[8,9].

The choice of enzymatic method, rather than chemical method for the synthesis of 5'-monophosphate, was based on the following considerations: Use of phosphoryl chloride with 2'-deoxynucleosides gives 3'-, as well as 5'-monophosphate[10,11]. Although the yield of 5'-monophosphate was increased by using deoxynucleosides with protected 3'-OH group, or by using trialkyl phosphate as solvent, or both[12,13], this procedure was not adopted by us for phosphorylation of O-alkyl deriv-

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atives of nucleosides because of the reported instability of these derivatives towards acids[14]. Use of pyridine in these reactions to neutralize the liberated HCl leads to phosphorylation of both 3'- and 5'-positions as observed in case of deoxyguanosine[15]. However, Hall and Saffhill[16] were able to prepare the 5'-monophosphate by treating O^6-methyldeoxyguanosine and O^4-methylthymidine with phosphoryl chloride in trialkyl phosphate solvent by controlling experimental conditions. Enzymatic phosphorylation, on the other hand, is selective to 5'-position and hence does not require protection of 3'-OH group, operates under conditions at which O-alkyl nucleosides are stable, and gives high yields of 5'-nucleotides[17].

Synthesis of triphosphates was carried out by the procedure of Hoard and Ott[18] with the modification that the monophosphate used was in the triethylammonium form, which was directly obtained from the DEAE-Sephadex column. It was not necessary to convert the triethylammonium form into tri-n-butylammonium form of the monophosphate used in the original procedure of Hoard and Ott[18] (Scheme I).

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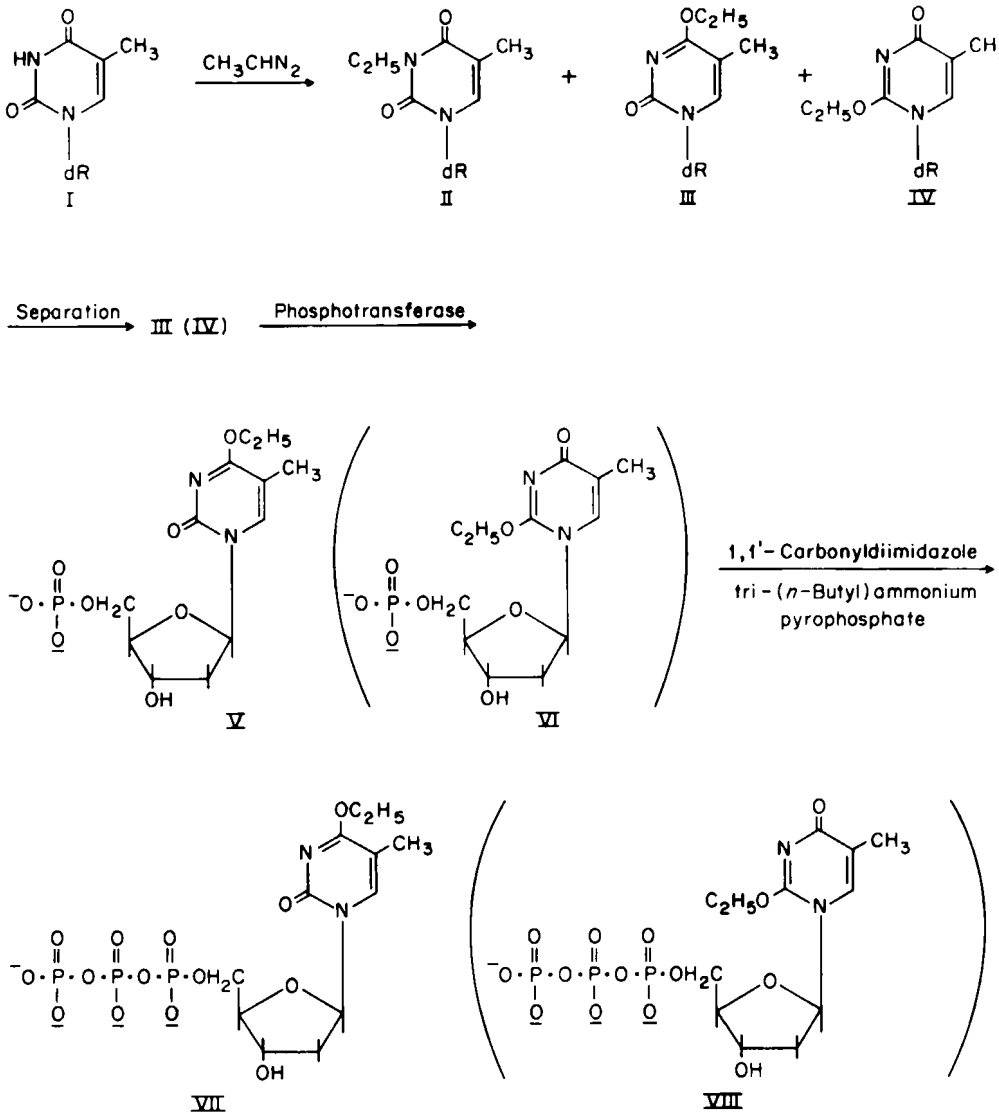
Materials. Thymidine, silica gel, bacterial alkaline phosphatase, 3'-nucleotidase and 5'-nucleotidase were obtained from Sigma Chemical Company. All organic solvents were obtained from Fisher Scientific Company. Pyridine was dried over calcium hydride and distilled. Dimethylformamide was dried over phosphorus pentoxide and distilled. Ethylurea was obtained from Aldrich Chemical Company. Triethylammonium bicarbonate was prepared by the procedure of Smith et al[19]. Carrot phosphotransferase was isolated according to the procedure of Strider et al[20] with the improvements suggested by Harvey et al[21]. Ultraviolet spectra were recorded on a Cary Model 14PM recording spectrophotometer.

Alkylation of thymidine. Ethereal solution of diazoethane was generated[22] from ethylnitrosourea prepared by the method of Amstutz and Myers[23]. Alkylation of the methanolic suspension of thymidine (12 mL, 0.08 M) with ethereal solution of diazoethane was carried out essentially by the procedure of Kusmierek and Singer[14]. After stirring overnight at room temperature, the organic solvents were evaporated in a rotary evaporator under vacuum, and loaded on a silica gel column (bed vol., 50 mL), and the products N-3-(II), O^4-(III) and O^2-ethylthymidine (IV) were collected by stepwise elution with 5%, 10% and 20% acetone in benzene respectively. Appropriate fractions were pooled and evaporated



SCHEME I

to dryness. The yields of II, III and IV were 90, 35, and 41 mg respectively. The compounds were identified by comparing their λ_{max} in water with the literature values (N^3-methylthymidine[24] 267 nm, $\underline{O}^4$-ethylthymidine[14], 278 nm, and $\underline{O}^2$-ethylthymidine[14] 226, 255 nm).

Synthesis of 5'-monophosphates. The 5'-monophosphate V (or VI) was prepared as follows: $\underline{O}^4$-ethylthymidine III (or $\underline{O}^2$-ethylthymidine IV)

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(0.1 mmole) and 4-nitrophenyl phosphate (1 mmole) were dissolved in 10 mL of 0.2 M sodium acetate, pH 5.0, 0.1 mL of carrot phosphotransferase[25] solution (1 mg protein per mL) was added, and the mixture was incubated at 37°C for 24 hrs. The progress of reaction was followed by TLC and Aminex A-6 column chromatography as follows: (1) Aliquots of reaction mixture (10-20 μL) at different time intervals were spotted on cellulose plate and developed by ethanol:1.0 M sodium acetate, pH 7.5 (7:3 v/v). In addition to bands for $\underline{O}^4$-ethylthymidine (R_f = 0.94) and 4-nitrophenyl phosphate (R_f = 0.35), the reaction mixture showed a band at 4-nitrophenol position (R_f = 0.98) and another new band (R_f = 0.43) presumably due to the formation of 5'-monophosphate. (2) A blank containing $\underline{O}^4$-ethylthymidine, 4-nitrophenyl phosphate, and sodium acetate buffer with the same concentrations of the compounds as in the reaction mixture, but containing no phosphotransferase, was injected into Aminex A-6 column (24 X 0.63 cm dia.) and eluted with 0.1 M ammonium borate, pH 7.5[26], at a flow rate of 0.31 mL/min. An equal volume of reaction mixture was also injected. The reaction mixture showed a smaller peak for $\underline{O}^4$-ethylthymidine (retention time, 52 min), and a new peak at monophosphate position, due to formation of V (Fig. 1). Detection of VI in the reaction mixture was done by the same procedures.

The product V (or VI) was isolated as follows: The reaction mixture was cooled in ice and repeatedly extracted with equal volumes of ether until the ether layer on treatment with sodium hydroxide solution did not impart a yellow color to the latter, indicating removal of 4-nitrophenol. The aqueous layer was diluted with water to 10 mL and loaded on an AG 1-X8 (anion-exchanger) column (HCO_3^- form, 11 X 1.5 cm dia.) maintained at 4°C. After eluting the unreacted compound III (or IV) with water, the product V (or VI) was eluted with a gradient of 0.0-1.0 M triethylammonium bicarbonate, pH 7.5 (500 mL). The unreacted 4-nitrophenyl phosphate was retained in the column and was eluted only with high salt concentration. The fractions containing O^4-ethylthymidine 5'-monophosphate, V (or O^2-ethylthymidine 5'-monophosphate, VI) were pooled, and repeatedly evaporated from 50% ethanol to remove triethylammonium bicarbonate. Yield of V was 54% of theory (VI, yield, 60%) as calculated from the following molar extinction coefficients; $\lambda_{max}^{H_2O}$ = 280 nm (ε_{280}, 6100) for V, $\lambda_{max}^{H_2O}$ = 257 (ε_{257}, 9200), 227 nm for VI); phosphorus:nucleoside ratio,

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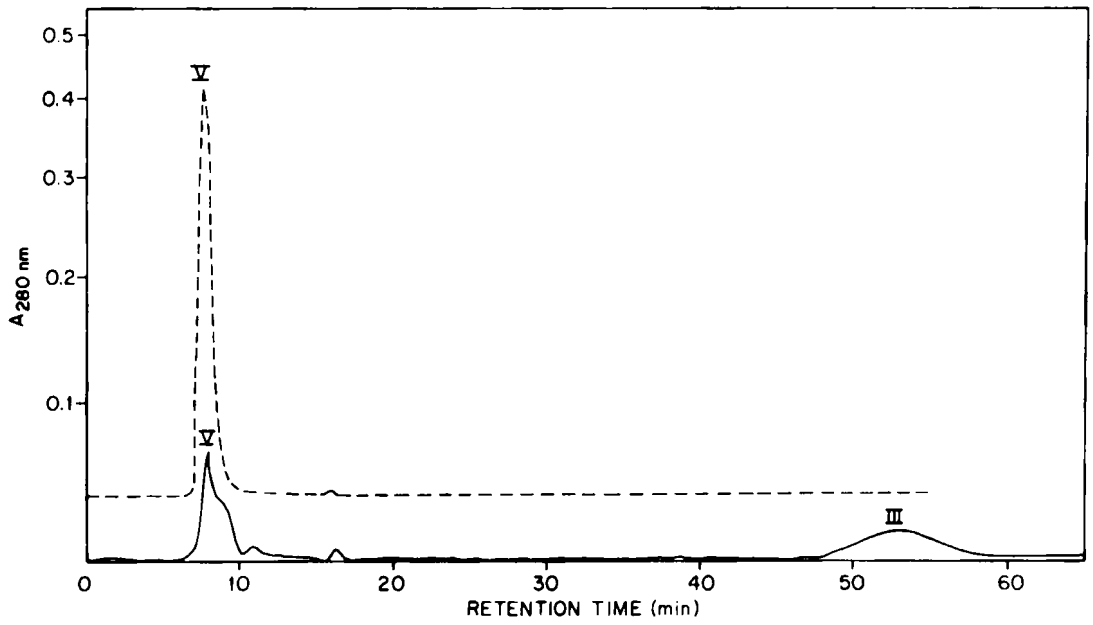

FIG. 1. Cleavage of Phosphate Group in V. Solution of V (70 µL) (3.7 A_{278} units) was treated with 10 mg/mL solution of bacterial alkaline phosphatase (5 µL) and 1 M tris, pH 8.0 (5 µL), and the mixture was incubated at 37°C for 1 hr. The whole mixture was passed through an Aminex A-6 column (24 X 0.63 cm), and eluted with 0.1 M ammonium borate, pH 7.5, at room temperature at a flow rate of 0.31 mL min. Degradation of V by phosphatase produced III (50-55 min peak) (solid line). Blank was prepared without addition of phosphatase (dotted line).

as determined by the method of Hurst and Becking[27], was found to be 1.05:1 for V (1.1:1 for VI).

Synthesis of 5'-triphosphates. Purified O^4-ethylthymidine 5'-monophosphate V (or O^2-ethylthymidine 5'-monophosphate VI) (2.7 µmole), as triethylammonium salt (as obtained from the Dowex 1-X8 column), was made anhydrous by repeated evaporation from 1 mL portions of anhydrous pyridine and then from 1 mL portions of anhydrous dimethylformamide (DMF), and finally suspended in 0.25 mL of anhydrous DMF, and then treated with 0.125 mmole of 1,1'-carbonyldiimidazole in 0.25 mL of anhydrous DMF, and stirred for 4 hrs at room temperature in a 5 mL round-bottomed flask fitted with calcium chloride drying tube. Excess of 1,1'-carbonyldiimidazole was destroyed with 8.3 µL of anhydrous methanol and stirred for another 30 min. The mixture was then treated with

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anhydrous tri-n-butylammonium pyrophosphate (0.125 mmole in 125 mL of anhydrous DMF), and the reaction was allowed to proceed for 24 hrs[18] at room temperature. After removal of the precipitate by centrifugation, the supernatant was evaporated to dryness under vacuum, and purified by DEAE-Sephadex A-25 column (26 X 1 cm dia., HCO_3^- form) by eluting with a gradient of 0-0.5 M triethylammonium bicarbonate, pH 7.5 (400 mL). The appropriate fractions containing O^4-ethylthymidine 5'-triphosphate VII (or O^2-ethylthymidine 5'-triphosphate VIII) were pooled, and repeatedly evaporated under vacuum from 50% ethanol to remove triethylammonium bicarbonate. Yield of VII, 22% of theory (yield of VIII 40% of theory) as calculated from the following molar extinction coefficients; $\lambda_{max}^{H_2O}$ = 278 nm (ε_{278}, 6100) for VII ($\lambda_{max}^{H_2O}$ = 255 (ε_{255}, 9200), 226 nm for VIII); phosphorus:nucleoside ratio = 3.1 for VII (3.1 for VIII).

Monitoring of the progress of the reaction and detection of the product (VII) in the reaction mixture were carried out as follows: (1) The reaction mixture (5 µL) was spotted on polyethyleneimine impregnated cellulose plate and developed with 1.0 M LiCl[28]. In addition to the band for the unreacted compound IV (R_f = 0.8), another band (R_f = 0.1) was seen in the triphosphate region, primarily due to the formation of VII. (2) The reaction mixture, 12 µL, was injected into an Aminex A-28 column (2.0 X 0.65 cm dia.), and eluted with a linear gradient (0.025-0.5 M) of citrate buffer, pH 8.4, at a flow rate of 0.1 mL/min. Additional peak was found in the triphosphate region (retention time, 52 min), due to formation of VII.

Enzymatic hydrolysis of triphosphates. A solution of VII (70 µL, 3.7 A_{278} units) was treated with 10 mg/mL solution of potato apyrase (5 µL), 1 mM $MgCl_2$ (5 µL) and 1 M Tris, pH 8.0 (5 µL), and the mixture was incubated at 37°C for 1 hr. A 10 mg/mL solution of bacterial alkaline phosphatase (5 µL) was then added and incubated again at 37°C for 1 hr. The whole mixture was analyzed by chromatography on Aminex A-6 column as described in the legend to Fig. 1. A blank sample was run without addition of enzymes. The chromatogram clearly indicated the formation of III by hydrolysis of VII. The product VII was also characterized in a similar fashion by hydrolysis to IV with potato apyrase and bacterial alkaline phosphatase.

Results and Discussion. The ethylated products of thymidine were eluted from silica gel column in the following order: N-3-ethylthymi-

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dine (II, $\lambda_{max}^{H_2O}$, 267 nm), $\underline{O}^4$-ethylthymidine (III, $\lambda_{max}^{H_2O}$, 278 nm) and $\underline{O}^2$-ethylthymidine (IV, $\lambda_{max}^{H_2O}$, 255,266 nm) and were identified by thin layer chromatography and spectral data[14].

The presence of phosphate group in the 5'-monophosphate, V, was shown by the degradation of V to the nucleoside III by bacterial alkaline phosphatase (Fig. 1). It is important to note that the compound V was degraded to III by treatment with 5'-nucleotidase, but not by 3'-nucleotidase, showing the position of phosphate group in V. Phosphorus: nucleoside ratio was found to agree with the theoretically expected value of 1:1, showing V to be a monophosphate.

Experiments with VI produced similar results.

Chromatography of the reaction mixture containing triphosphate on the DEAE-Sephadex column showed three UV-absorbing peaks. The third peak was found to be the triphosphate with phosphorus:nucleoside ratio of 3.1:1, the first peak was identified as the monophosphate, and the second peak was found to have phosphorus:nucleoside ratio of 2.1:1 which was not further characterized.

The presence of the triphosphate group in VII was indicated by retention time in HPLC (Aminex A-28, C-18 μBondapak), TLC (polyethyleneimine impregnated cellulose, 1 0 M LiCl), salt concentration required for elution from DEAE-Sephadex column. Phosphorus:nucleoside ratio was found to be in agreement with the theoretically expected value of 3.0 for VII. The ultraviolet spectrum of VII was in agreement with that of $\underline{O}^4$-ethylthymidine. The compound VII was also degraded with potato apyrase and bacterial alkaline phosphatase to produce III, further confirming the structure of VII. Analysis of VI by HPLC showed the compound to be 97% pure. Analysis of VII in the same way produced similar results.

The present study affords a method for synthesis, purification and identification of $\underline{O}$-ethylated pyrimidine nucleoside triphosphates on microscale; and by using radio-labeled thymidine as the starting material, it should be possible to synthesize the labeled ethylated triphosphate.

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