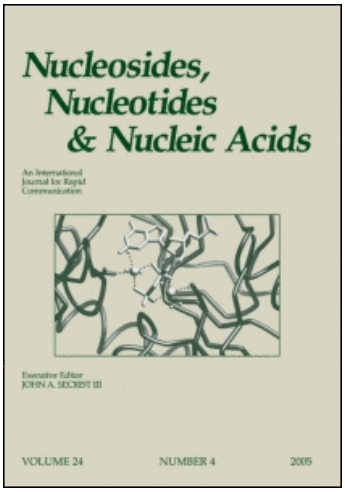

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The Large Scale Synthesis and Chromatographic and Spectral Properties of a Series of Alkylated Thymine and Uracil-Containing Nucleosides. O^2-, 3- and O^4-Alkylpyrimidine Derivatives

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THE LARGE SCALE SYNTHESIS AND CHROMATOGRAPHIC AND SPECTRAL PROPERTIES OF A SERIES OF ALKYLATED THYMINE AND URACIL-CONTAINING NUCLEOSIDES. O^2-, 3- AND O^4-ALKYLPYRIMIDINE DERIVATIVES

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SUMMARY

The syntheses of the O^2-, 3- and O^4-alkyl- (methyl-, ethyl-, n-propyl- and n-butyl-) deoxythymidines are described along with those of the methyldeoxyuridines and some alkyl-5-methyluridines. A simple direct alkylation procedure using the appropriate diazoalkane is used, followed by efficient chromatographic separations. These allow the compounds to be synthesised and purified in gram quantities if necessary. The chromatographic and UV spectral properties of the compounds are summarised together with their proton magnetic resonance spectra.

INTRODUCTION

Alkylating carcinogens and mutagens react with DNA to form a wide range of alkylated bases within the DNA with the exact proportions and amounts of the various reaction products depending upon the alkyl group and the type of alkylating agent used[1]. Amongst the products that have been identified in enzyme hydrolysates of alkylated DNA are O^2-, 3- and O^4-alkyldeoxythymidines (structures I, II and III, Fig. I). During DNA synthesis O^4-methyl- and O^4-ethyldeoxythymidines are potential promutagenic lesions[2,3,4] and O^2-methyl- and O^2-ethyldeoxythymidines could be promutagenic during RNA synthesis[4,5]. The methyl and ethyl derivatives of deoxythymidine and uridine have previously been prepared either by lengthy procedures involving glycosidic bond synthesis or by direct alkylation of the parent nucleoside[2,6,7,8]. In the latter case the purification procedures used have not been readily adaptable to large scale preparations. Recently O^2- and O^4-iso-propyldeoxythymidines have been

prepared by reacting iso-propyl iodide with deoxythymidine in the presence of silver oxide[9], and the compounds have then been converted to the methyl derivatives by reaction with sodium methoxide but the purification procedures used were not readily adaptable to large scale preparations.

We have found it necessary to prepare several alkyl derivatives of deoxythymidine and related nucleosides as reference and marker compounds in radioactive tracer studies on the alkylation of DNA[10], for catabolic studies on alkylated nucleic acid components[11], for studies on direct incorporation into cellular DNA[8,12] and as antigens and competitive inhibitors in the development of sensitive radioimmunoassay[13,14] methods for the quantitation of alkylated bases formed in DNA. A series of alkylated (methyl-, ethyl-, propyl- and butyl-) nucleosides (Fig. 1) has been prepared by direct alkylation of the parent nucleoside with the appropriate diazoalkane. The products have been separated by chromatography on Dowex-50 or by preparative reverse phase high performance liquid chromatography (HPLC). The paper chromatographic properties of the methyl- and ethyl-deoxythymidines were as previously reported, as were the UV-spectra[7] at neutral, acidic and alkaline pH. All of the other $\underline{O}^2$-, 3- and $\underline{O}^4$-alkyl derivatives of deoxythymidine and 5-methyluridine gave UV-spectra similar to the corresponding methyldeoxythymidine. The $\underline{O}^2$-, 3- and $\underline{O}^4$-methyldeoxyuridines gave spectra similar to those already described for the appropriate methyluridine[7].

The glycosidic bond of $\underline{O}^2$-alkyldeoxythymidines is unstable under acidic conditions[15] and we have prepared the $\underline{O}^2$-alkylthymines (IV) in good yields from the corresponding deoxynucleoside by depyrimidination in 0.1 M HCl at 80°. All of the $\underline{O}^2$-alkylthymines gave UV-spectra similar to those reported for $\underline{O}^2$-methylthymine[15].

Although the synthesis and some of the properties of several of these compounds have previously been reported they have sometimes only been given with brief details, which in many cases were incomplete particularly with regard to the HPLC data and extinction coefficients. We believe that a detailed description of the synthetic methods and of the properties of the compounds would make them more accessible to workers in many fields. Unlike the methods of purification involving paper and thin layer chromatography (TLC) used previously for the purification of the methyl and the ethyl derivatives of deoxythymidine and uridine the separations described here may conveniently be used to prepare the compounds in gram quantities. The syntheses have the advantage of being simple, one step,

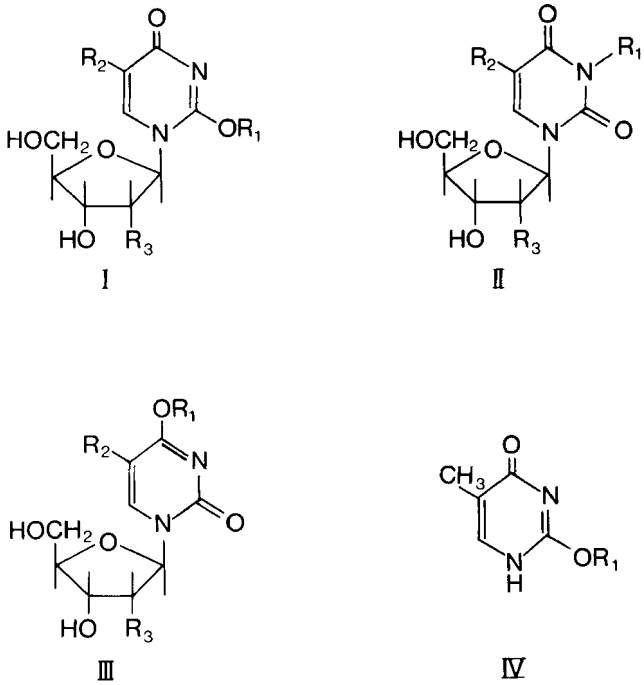

FIGURE 1

direct alkylations followed by efficient separation techniques that do not require expert synthetic expertise. Although the methyldeoxythymidines could be separated by preparative reverse phase HPLC the low yields of $\underline{O}^2$- and $\underline{O}^4$-methyldeoxythymidines from deoxythymidine mean that relatively large amounts of the parent nucleoside must to be methylated and chromatographed. Such separations are more readily achieved using a high capacity Dowex 50 column which allows the products from several grams of deoxythymidine to be separated each time. For smaller scale preparations, HPLC separation should be preferable. Although the ethyl derivatives could also be chromatographed on this column the retention times for the n-propyl- and n-butyl- derivatives were too great and the peaks too broad to give good separations. In these cases preparative HPLC was used. The chromatographic properties of the compounds in the analytical systems that have been used in our laboratory, together with their melting points, are summarised in Table I. The UV-spectral properties are summarised in Table II and selected proton magnetic resonance (pmr) data is given in Table III.

TABLE I Chromatographic Properties of Alkylpyrimidine Nucleosides

	Melting Point °C	HPLC Retention[a] mins	Aminex A6[b] Retention mins	Dowex 50 Retention[c] fraction	TLC[d] Rf	Paper[e] Rf
dT		12.1	26.8	87	0.32	0.47
dU		7.8		83	0.20	0.41
rT		9.3		86	0.11	
O^2-MedT	143-4	13.3	30.2	63	0.05	0.58
3-MedT	-	16.9	32.9	73	0.51	0.69
O^4-MedT	168-9	19.1	46.3	94	0.20	0.69
O^2-MedU	133-4	11.2		61	0.02	0.71
3-MedU	144-6	12.8		70	0.39	0.78
O^4-MedU	137-8	16.9		93	0.22	0.79
O^2-MerT	164-6	12.4		63	<0.01	
3-MerT	147-8	14.6		72	0.37	
O^4-MerT	-[f]	17.0		93	0.15	
O^2-EtdT	163-5	17.8	42.2	72	0.10	0.71
3-EtdT	132-3	19.3	55.1	80	0.59	0.80
O^4-EtdT	186-7	25.3	77.1	107	0.37	0.79
O^2-PrdT	154-6	23.3	48.8		0.19	
3-PrdT	166-8	26.6	66.6		0.63	
O^4-PrdT	142-3	33.2	92.6		0.41	
O^2-BudT	137-9	28.8	55.9		0.18	
3-BudT	158-9	30.4	70.0		0.69	
O^4-BudT	146-7	39.3	134.6		0.36	
O^2-BurT	171-2	26.9			0.16	
3-BurT	156-8	29.1			0.53	
O^4-BurT	161-2	36.0			0.39	
O^2-MeT	-[f]	12.1			0.39	
O^2-EtT	-[f]	14.7			0.44	
O^2-PrT	-[f]	17.8			0.51	
O^2-BuT	181-3	25.1			0.57	

a Partisil-10 ODS-II (25 x 0.5 cm). See text for conditions.
b Aminex A-6 (25 x 1 cm) eluting with 10 m$\underline{M}$ NH_4HCO_3 with a flow of 0.5 ml/min at 50°.
c Dowex-50 (90 x 1.6 cm) eluting with 1 m$\underline{M}$ KP_i pH7 with a flow of 20 ml/hr collecting 10 ml fractions.
d Acetone/benzene - 2/1 on Keiselguhr GF_{254} plates.
e Butan-1-ol/ethanol/water - 80/10/25.
f Compound was not isolated as a crystalline compound. See text.

METHODS

Thin layer chromatography (TLC) and HPLC were used as analytical techniques to follow the reactions and purification procedures and to check the purity of the final products. TLC was carried out on Keiselguhr GF254 plates (10 x 5 cm) using acetone/benzene (2/1). Paper chromatography was carried out on Whatman No. 1 paper using butan-1-ol/ethanol/water

TABLE II UV Spectra of Alkylpyrimidine Nucleosides

	pH 7[a]				pH 1[b]				pH 13[c]			
	Maximum		Minimum		Maximum		Minimum		Maximum		Minimum	
	λ[d]	ε[e]	λ	ε	λ	ε	λ	ε	λ	ε	λ	ε
O2-MedT	255	9.50	234	6.34	258	9.07	235	5.21	255	6.63	236	4.85
	228	6.40	216	4.52	231	6.17	216	4.48				
3-MedT	266	8.68	236	2.22	266	8.58	236	2.27	267	8.93	237	2.78
O4-MedT	280	6.68	240	0.90	280	7.00	240	0.94	281	6.46	242	1.14
O2-MedU	251	9.67	236	6.42	253	9.24	236	6.86	251	9.39	238	8.07
	225	7.20	213	4.54	224	8.26	212	6.89				
3-MedU	261	7.12	231	1.50	261	6.84	232	1.87	261	7.16	236	3.27
O4-MedU	273	6.24	236	0.74	275	6.15	237	1.20	273	6.31	239	1.46
O2-MerT	256	9.26	234	6.25	258		235		255		237	
	229	6.33	216	4.38	231		216					
3-MerT	266	8.72	236	2.41	266		236		267		237	
O4-MerT	280	6.52	240	0.83	280		240		281		242	
O2-EtdT	256	9.32	235	6.40	258		235		255		236	
	229	6.48	216	4.31	233		217					
3-EtdT	267	8.40	237	2.35	266		236		267		237	
O4-EtdT	280	6.52	240	0.83	280		240		281		242	
O2-PrdT	256	9.40	228	6.43	259		233		257		238	
	230	6.35	216	4.50	232		219					
3-PrdT	266	8.52	236	2.34	266		236		267		237	
O4-PrdT	280	6.43	240	0.87	280		240		281		243	
O2-BudT	256	9.67	229	6.33	259		236		256		239	
	226	6.35	216	4.44	230		219					
3-BudT	267	8.40	237	2.35	267		237		267		239	
O4-BudT	280	6.55	240	0.90	281		240		281		243	
O2-BurT	256	9.32	230	6.33	258		236		257		240	
	226	6.32	216	4.62	230		219					
3-BurT	267	8.43	236	2.23	267		236		267		238	
O4-BurT	280	6.32	240	0.83	281		240		281		242	

a Determined in 10 m<u>M</u> K Phosphate, pH 7.
b Determined in 0.1 <u>M</u> HCl.
c Determined in 0.1 <u>M</u> NaOH.
d Wavelength, nm.
e Extinction Coefficient $x10^{-3}$.

(80/10/25) by the descending technique. The compounds were detected under UV-light at 254 nm. Analytical HPLC was carried out on Partisil-10 ODS-II (25 x 0.5 cm) eluting with a linear gradient of (5 - 50) aqueous methanol over a period of 30 minutes then isocratically at the higher concentration with a flow of 1.5 ml/min. Dowex 50 chromatography (90 x 1.6 cm, potassium form) was carried out eluting with 1 m<u>M</u> potassium phosphate pH 7 at a flow of 50 ml/hr collecting 10 minute fractions. Aminex A-6 chromatography (25 x 1 cm, ammonium form) was carried out eluting with 10 m<u>M</u> ammonium

TABLE III Details of the PMR Spectra of Alkyldeoxythymidines

	5-Me (or 5-H[a])		6-H		N or O Alkyl	
dT	2.44(s[b])	(3H[c])	8.22(s)	(1H)		
O^2-MedT	2.98(s)	(3H)	8.90(s)	(1H)	5.04(s)	(3H)
3-MedT	2.96(s)	(3H)	8.70(s)	(1H)	4.32(s)	(3H)
O^4-MedT	3.02(s)	(3H)	9.00(s)	(1H)	5.02(s)	(3H)
O^2-MerT	2.96(s)	(3H)	8.88(s)	(1H)	5.00(s)	(3H)
3-MerT	2.96(s)	(3H)	8.74(s)	(1H)	4.32(s)	(3H)
O^4-MerT	2.90(s)	(3H)	9.04(s)	(1H)	5.06(s)	(3H)
O^2-MedU	7.24(d)	(1H)	9.10(d)	(1H)	5.12(s)	(3H)
3-MedU	6.98(d)	(1H)	8.88(d)	(1H)	4.30(s)	(3H)
O^4-MedU	7.32(d)	(1H)	9.16(d)	(1H)	5.14(s)	(3H)
O^2-EtdT	2.86(s)	(3H)	8.90(s)	(1H)	2.12(t)	(3H)
					3.36(m)	(2H)
3-EtdT	2.88(s)	(3H)	8.74(s)	(1H)	2.10(t)	(3H)
					2.84(m)	(2H)
O^4-EtdT	2.90(s)	(3H)	8.98(s)	(1H)	2.10(t)	(3H)
					3.44(m)	(2H)
O^2-PrdT	2.90(s)	(3H)	8.86(s)	(1H)		
3-PrdT	2.90(s)	(3H)	8.78(s)	(1H)		
O^4-PrdT	2.94(s)	(3H)	8.94(s)	(1H)		
O^2-BudT	2.92(s)	(3H)	8.90(s)	(1H)		
3-BudT	2.88(s)	(3H)	8.76(s)	(1H)		
O^4-BudT	2.96(s)	(3H)	8.94(s)	(1H)		
O^2-BurT	2.94(s)	(3H)	8.82(s)	(1H)		
3-BurT	2.86(s)	(3H)	8.76(s)	(1H)		
O^4-BurT	2.94(s)	(3H)	8.92(s)	(1H)		

a For deoxyuridine derivatives.
b (s) singlet, (d) doublet, (t) triplet, (m) multiplet.
c Integrated number of protons.
d The pmr spectra of the higher derivatives (propyl and butyl) could not be fully analysed.

carbonate (unbuffered) at a flow of 30 ml/hr at 80°. The column eluates were monitored at 254 nm. UV-spectra were determined in 10 mM potassium phosphate pH 7, 0.1 M HCl and 0.1 M KOH. Pmr spectra were determined in deuterium oxide using tert-butanol as internal standard with a Hitachi/Perkin-Elmer R600 machine operating at 60 MHz. Satisfactory elemental (C, H and N) analyses have been obtained for all new compounds.

N-Alkyl-N-nitrosoureas. N-Ethyl-, N-propyl- and N-butyl- N-nitrosoureas were prepared by nitrosation of the corresponding N-alkylurea by the method described by Vogel[16] for the sythesis of N-methyl-N-nitrosourea. The

products were filtered off, washed with ice-cold water and dried in vacuo over P_2O_5 at room temperature. They were stored -25°.

Diazoalkanes. Ethereal diazomethane was prepared from Diazald (N-methyl-N-nitroso-p-toluenesulphonamide) as described by Vogel[16]. Ethereal diazoethane was prepared by a modification of the method of Robbins et al[17]. N-Ethyl-N-nitrosourea (3 g) was added to a rapidly stirred mixture of 40% aqueous KOH (30 ml) and ether (100 ml) at 0°. After 10 minutes the two layers were allowed to separate and the upper yellow ether layer was removed. The method used for the preparation of diazoethane did not prove satisfactory for the preparation of diazopropane and diazobutane because the yields of diazoalkane were very low and the ether layers were found to contain large amounts of unconverted nitrosourea. Instead, the nitrosourea (n-propyl- or n-butyl-) (3.5 g) was dissolved in ether (175 ml) and cooled in ice. 10% Methanolic KOH (70 ml) was added and the mixture stirred for 10 minutes at 0° after which water (250 ml) was added and the orange ether layer, containing the diazoalkane, was allowed to separate and was removed as soon as possible. All diazoalkanes were kept over KOH pellets at 0° and used as soon as possible after preparation.

Alkylation of Nucleosides. The nucleoside was dissolved in methanol (30 ml per g of nucleoside) and the solution cooled in ice. An ethereal solution of the appropriate diazoalkane was added portionwise at a rate sufficient to maintain a pale yellow colour until the colour persisted. After standing in ice for a further hour, TLC and HPLC showed that all of the starting nucleoside had reacted and that three products had been formed. These corresponded to the O^2-, 3- and O^4-alkyl derivatives as shown by their UV-spectra[7]. For all preparations, the HPLC retention times increased in the order of O^2- < 3- < O^4-alkylderivatives whilst the TLC Rfs increase in the order O^2- < 3 < O^4-. The solvents were removed in vacuo and the residue was dissolved in water and purified as described below for individual compounds.

Methyldeoxythymidines[8,12] (I, II and III). Deoxythymidine (25 g) was methylated with diazomethane. The residue was dissolved in boiling water (150 ml) and the 3-methyldeoxythymidine crystallised on cooling. The crystals were filtered off when HPLC and TLC of the mother liquors showed them to contain residual 3-methyldeoxythymidine along with O^2-methyldeoxythymidine and O^4-methyldeoxythymidine. The mixture was divided into 6 aliquots which were in turn chromatographed on Dowex 50,

potassium form (90 x 3.2 cm), eluting with water at a flow of 300 ml/hr and collecting fractions of 13 ml. O^2-methyldeoxythymidine eluted in fractions 24 - 31, 3-methyldeoxythymidine in fractions 34 - 44 and O^4-methyldeoxythymidine in fractions 55 - 68. Those fractions containing O^2- and O^4-methyldeoxythymidines were pooled separately and evaporated to dryness *in vacuo*, (bath temperature <35°). Both compounds crystallised on the addition of ethyl acetate. Yields, O^2-methyldeoxythymidine - 0.879 g (3.3%), 3-methyldeoxythymidine - 18.67 g (70.6%) and O^4-methyldeoxythymidine - 1.714 g (6.5%). The yield of 3-methyldeoxythymidine could be increased by recovery from fractions 34 - 44 following chromatography.

Methyldeoxyuridines[12] (I, II and III). Deoxyuridine (3 g) was methylated with diazomethane. The 3-methyldeoxyuridine could not be induced to crystallise (cf 3-methyldeoxythymidine) without purification. The residue was divided into 3 aliquots which were chromatographed on Dowex 50 as described for the methyldeoxythymidines. The compounds eluted in the order O^2-, 3- and O^4-methyldeoxyuridine and, after removal of water *in vacuo*, the compounds crystallised on the addition of ethyl acetate. Yields, O^2-methyldeoxyuridine - 0.072 g (2.3%), 3-methyldeoxyuridine - 2.79 g (83.7%) and O^4-methyldeoxyuridine - 0.092 g (2.8%).

Methyl-5-methyluridines[14] (I, II and III). 5-methyluridine (1 g) was methylated with diazomethane. The mixture was chromatographed in two parts on Dowex 50 as already described. The compounds eluted in the order O^2-, 3- and O^4,5-dimethyluridine. After pooling those fractions containing the purified compounds they were evaporated to dryness *in vacuo* and the O^2,5- and 3,5-dimethyluridines crystallised on addition of ethyl acetate. The O^4-methylribonucleoside could not be induced to crystallise from a variety of solvents but it was obtained as a chromatographically homogeneous glass by lyophilisation of an aqueous solution. Yields, O^2,5-dimethyluridine - 0.036 g (3.4%), 3,5-dimethyluridine - 0.68 g (64.5%) and O^4,5-dimethyluridine - 0.045 g (4.3%).

Ethyldeoxythymidines[13] (I, II and III). Deoxythymidine (0.5 g) was ethylated with diazoethane and the reaction products were chromatographed in two parts on Dowex 50 as described above. Alternatively, aliquots could be purified by preparative HPLC on Partisil-10 ODS-II (50 x 1 cm) eluting with aqueous methanol at a flow of 7 ml/min. Methanol (15%) was used until the O^2- and 3-ethyldeoxythymidines had eluted when the methanol concentration was increased to 35% to elute the O^4-ethyldeoxythymidine.

After evaporation to dryness the compounds crystallised on the addition of ethyl acetate. Yields, O^2-ethyldeoxythymidine - 0.091 g (8.2%), 3-ethyldeoxythymidine - 0.473 g (42.4%) and O^4-ethyldeoxythymidine - 0.135 g (12.1%).

n-Propyldeoxythymidines[13] (I, II and III). Deoxythymidine (0.25 g) was propylated with diazopropane. The products were purified by preparative HPLC on Partisil-10 ODS-II (50 x 1 cm) eluting with aqueous methanol at a flow of 7 ml/min. Methanol (25%) was used until the O^2- and 3-n-propyldeoxythymidines had eluted when the concentration was increased to 50% to elute the O^4-n-propyldeoxythymidine. After evaporating to dryness the compounds crystallised on the addition of ethyl acetate. The compounds could be recrystallised from water, ethanol or acetone. Yields, O^2-n-propyldeoxythymidine - 0.045 g (15.3%), 3-n-propyldeoxythymidine - 0.117 g (39.9%) and O^4-n-propyldeoxythymidine - 0.026 g (8.9%).

n-Butyldeoxythymidines[10,13] (I, II and III). Deoxythymidine (0.5 g) was butylated with diazobutane. The products were purified in 4 aliquots by preparative HPLC on Partisil-10 ODS-II eluting with aqueous methanol at a flow rate of 7 ml/min. Methanol (35%) was used until the O^2- and 3-n-butyldeoxythymidines had eluted when the concentration was increased to 50% to elute the O^4-n-butyldeoxythymidine. After evaporating to dryness the compounds crystallised on addition of ethyl acetate. Yields, O^2-n-butyldeoxythymidine - 0.121 g (20.0%), 3-n-butyldeoxythymidine - 0.085 g (13.8%) and O^4-n-butyldeoxythymidine - 0.185 g.

n-Butyl-5-methyluridines[13] (I, II and III). 5-methyluridine (0.5 g) was butylated with diazobutane and the products purified as described above for the n-butyldeoxythymidines. Yields, O^2-n-butyl-5-methyluridine - 0.139 g (22.8%), 3-n-butyl-5-methyluridine - 0.112 g (18.4%) and O^4-n-butyl-5-methyluridine - 0.167 g (27.4%).

O^2-Alkylthymines (IV). The appropriate O^2-alkyldeoxythymidine was dissolved in 0.1 M HCl at a concentration of 10 mg/ml and the solution heated to 80° for 10 minutes. After cooling in ice the solution was cooled and then neutralised by the addition of 1 M K_2HPO_4 (0.25 ml/ml of 0.1 M HCl. The compounds were purified by preparative HPLC on Partisil-10 ODS-II (50 x 1 cm) eluting with a linear gradient 5 to 50% aqueous methanol over a period of 60 minutes with a flow of 5 ml/min. Deoxythymidine and thymine were also formed as minor products. After pooling the fractions and evaporating to dryness the compounds could be crystallised by the addition of ethyl

acetate and recrystallised from water. Yields, O^2-Methylthymine - 0.042 g (76.8%) from 100 mg nucleoside). O^2-Ethyl and O^2-n-propylthymines were prepared from 5 mg nucleoside and the products were not isolated as crystalline solids. The yields (based on optical measurements using the extinction coefficients of the methyl and n-butyl derivatives were 65% and 73% respectively. O^2-n-Butylthymine - 0.015 g (81.8%) from 30 mg nucleoside.

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