Methylation of Pyrimidine 2'-Deoxynucleosides with Trimethyl Phosphate

Toshizumi TANABE,* Kiyoshi YAMAUCHI, and Masayoshi KINOSHITA
Department of Applied Chemistry, Osaka City University, Sumiyoshi-ku, Osaka 558
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The reactions of thymidine, deoxyuridine, and deoxycytidine with trimethyl phosphate (TMP) were studied in a homogeneous water solution at 37—60 °C and pH 7—10. Three pyrimidine deoxynucleosides were methylated mainly at the N-3 position to afford their 3-methyl derivatives. The methylation rate at the N-3 position was found to be dependent on the pH of reaction medium. At pH 10, thymidine and deoxyuridine were methylated much faster than deoxycytidine, the tendency being reversed at pH 7.

Most chemical carcinogens such as polycyclic aromatics, aromatic amines, and azo compounds have recently been found to be activated *in vivo* by oxidizing enzymes to alkylate nucleic acids, causing mutagenic or carcinogenic effects.[1] Since intrinsic alkylating agents such as dialkyl sulfates, alkyl alkanesulfonates, and diazo alkanes also have mutagenicity or carcinogenicity, many studies have been carried out on their actions on nucleic acids and their components.[2-7]

We reported the alkylation of five main nucleic acid-bases with trialkyl phosphates such as trimethyl,[8] triethyl,[9] and triallyl phosphates[10] (TMP, TEP, and TAP). From the results, TMP and TEP appear to be suitable for the alkylation of nucleic acids and their components because of their high solubility in water and moderate reactivity which enables the alkylating reactions to take place homogeneously in an aqueous phase. Methylation study with TMP seems to be worthy of further studies, since many methylated nucleosides are present in nucleic acids from various sources.

We have studied the methylation of pyrimidine deoxynucleosides such as thymidine (**1**), deoxyuridine (**2**), and deoxycytidine (**3**) with TMP in homogeneous aqueous phase of pH 7—12 at 37 and 60 °C.

The characterization of the products from these reactions is given in the following. The effect of pH of reaction mixtures on methylation rate is also discussed.

Results and Discussion

The reactions were carried out at 37 and 60 °C by stirring a mixture of a deoxynucleoside and TMP in water at an appropriate pH. The products were isolated by extraction and column chromatography and identified by means of UV and NMR spectra. Melting point and R_f value were also employed for the identification of products. The yields of the products were determined by means of UV spectra. The results are summarized in Tables 1 and 2.

Thymidine (1) and Deoxyuridine (2). Most methylation studies on thymidine (**1**) so far reported were conducted using diazomethane in water–ether[3] or methanol–ether[2,5] solution. The results indicate that diazomethane methylated **1** at the N-3 position chiefly to afford 3-methylthymidine (**4**) with co-formation of a small amount of O^4-methylthymidine. Treatment of DNA with *N*-methyl-*N*-nitrosourea, a possible source of diazomethane *in vivo*, has been reported to afford 3-

Table 1. Methylation of thymidine (**1**) and deoxyuridine (**2**)[a]

Deoxynucleosides (dNu)	Temp (°C)	pH	Mole ratio (TMP/dNu)	Product	Yield (%)[b] 6 h	12 h	24 h	48 h
Thymidine(**1**)	37	7	15	3-Methylthymidine(**4**)	0	0	0	trace
1	37	8	15	**4**	13	17	22	26
1	37	9	15	**4**	24	34	50	61
1	37	10	15	**4**	35 (30)[c]	42 (43)	76 (63)	81 (81)
1	37	10	7.5	**4**	—	37	55	78
1	37	10	30	**4**	—	71	89	97
1	37	10	60	**4**	—	84	90	100
1	37	11	15	**4**	46	65	83	93
1	37	12	15	**4**	42	63	80	87
				3,O^5'-Dimethylthymidine	trace	trace	3	5
1	60	7	15	**4**	—	14	15	22
1	60	10	15	**4**	83	96	—	—
Deoxyuridine(**2**)	37	8	15	3-Methyldeoxyuridine(**5**)	—	11	16	23
2	37	9	15	**5**	16	28	47	71
2	37	10	15	**5**	37 (34)[c]	55 (53)	73 (72)	88 (88)
2	37	11	15	**5**	67	77	86	95

a) Reaction size: a deoxynucleoside (0.25 mmol) + TMP (1.9 mmol, 3.8 mmol, 7.5 mmol, or 14.3 mmol) + H_2O (5.0 ml) at 37 or 60 °C. b) Spectroscopic yield. c) The yields in parentheses are those of the reactions of **1** and **2** with TMP in the same vessels.

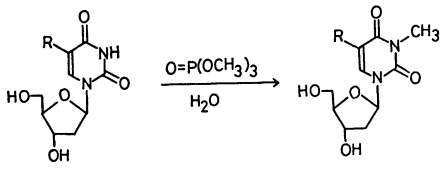
Table 2. Methylation of deoxycytidine (3)[a]

Temp (°C)	pH	Mole ratio (TMP/3)	Product	Yield (%)[b] 24 h	48 h	72 h
37	7	15	3-Methyldeoxycytidine(**6**)	4	9	13
37	7	30	**6**	12	15	25
37	7	60	**6**	17	23	30
37	10	15	**6**	4	6	7
			3-Methyldeoxyuridine(**5**)	0	trace	4
37	10	30	**6**	6	9	13
			5	0	1	4
37	10	60	**6**	13	18	21
			5	3	2	4
37	12	30	**5**	0	trace	1
			$O^{x'}$-Methyldeoxycytidine	1	4	5
60	7	15	**6**	37	59	68
60	10	15	**5**	33	53	63
			3,$O^{x'}$-Dimethyldeoxyuridine	0	4	5

a) Reaction size: deoxycytidine (0.25 mmol)+TMP (3.8 mmol, 7.5 mmol, or 14.3 mmol)+H_2O (5.0 ml) at 37 or 60 °C. b) Spectroscopic yield.

methyl- and O^4-methylthymidines in addition to several methylated purine nucleosides.[11] On the other hand, little is known about the alkylation of deoxyuridine (**2**) which is present merely as the precursor of **1** in living systems.

The present reactions of **1** with TMP in an aqueous phase gave the results as shown in Table 1. Methylation occurs at the N-3 position of **1** to afford 3-methylthymidine (**4**) as the sole product at pH 7—11. However, when the pH is raised to 12, thin-layer chromatography of the reaction mixture shows two spots. One product (low R_f) was isolated and identified as **4**, the other product being assigned as 3,$O^{5'}$-dimethylthymidine by comparison of its UV spectrum and R_f value with those of an authentic sample.



1: R=CH_3
2: R=H
4: R=CH_3
5: R=H

The methylation rate of **1**, particularly the formation of **4**, was found to be dependent on the pH of reaction medium. In the pH region 7—11 at 37 °C, the higher the pH, the faster the methylation rate. For instance, **4** was produced quantitatively in the reaction at pH 11, while the reaction hardly proceeded at pH 7. However, when the pH of reaction mixture was raised from 11 to 12, no noticeable increase in methylation rate was observed at the N-3 position of **1**. The results suggest that methylation of **1** occurs most likely in a bimolecular fashion between the anionic form of **1** and TMP, since the dissociation of the N-3 proton of **1** (pK_a:9.8)[12] gradually increases in the pH range 7—11. At pH 11, the N-3 proton dissociates nearly completely, the methylation rate at the N-3 position under present conditions becoming maximum. The pH change from 11 to 12 might not affect the methylation rate at the N-3 position of **1**.

The formation of a small amount of 3,$O^{5'}$-dimethylthymidine in the reaction at pH 12 may also be attributed to the dissociation of 5'-OH group (pK_a>13) to the oxido anion and its subsequent reaction with TMP.

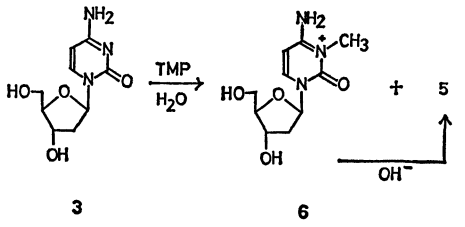
The rise in reaction temperature and increase in the mole ratio of TMP to **1** also accelerated the methylation rate (Table 1).

Methylation of deoxyuridine (**2**) with TMP gave almost the same result as that of methylation of **1**, **2** being methylated at the N-3 position to give 3-methyldeoxyuridine (**5**), whose yields increased gradually with the rise of pH of the reaction mixture. However, the reaction of TMP with a mixture of **1** and **2** at pH 10 showed that **2** is methylated at the N-3 position faster than **1**, probably because the pK_a value of the former is smaller, *e.g.* 9.2 and 9.8, respectively.[12]

Deoxycytidine (3). Several reports have appeared on the alkylation of cytidine and deoxycytidine (**3**).[3-7] Treatment of cytidine with diazomethane in water–ether[3] and dimethyl sulfate in DMF[4] caused methylation at the N-3 position of cytosine ring. However, **3** remains unchanged in the reaction with diazomethane in methanol–ether solution.[5] Sun and Singer studied methylation and ethylation of cytidine and **3** in detail using alkyl iodide, ethyl methanesulfonate, and diethyl sulfate, and observed the formation of 3-alkyl derivatives along with a small extent of alkylation at exocyclic 4-amino group of cytosine ring.[6]

In the present reactions, methylation took place smoothly at the N-3 position to afford 3-methyldeoxycytidine (**6**) at pH 7, while the reaction at pH 10 gave a mixture of **6** and 3-methyldeoxyuridine (**5**) (Table 2).

The production of **5** under alkaline conditions can be attributed to alkaline hydrolysis of **6**, since the analogous compound, 1,3-dimethylcytosine was trans-



formed into 1,3-dimethyluracil and the conversion of **3** into **2** was not observed under similar conditions. Since the deprotonation of the pyrimidine ring of **3**, established to exist in the amino form,[13] does not take place under the present conditions, the formation of **6** would occur most likely by the attack of TMP on the neutral N-3 position of the amino form of **3**.

The methylation rate at the N-3 position showed a slight dependence on the pH of reaction medium, which is, however, opposite to that observed in the methylation of **1**. That is, the methylation rate decreased with the increase of pH. Especially, at pH 12, methylation hardly occurred at the N-3 position of **3**, while deoxyribose moiety was methylated in a small amount. A similar trend of **3** or cytidine to resist methylation at the N-3 position under alkaline conditions has recently been reported by Kuśmierek *et al.*[7]

The above reactions thus reveal that **1** and **2** more easily undergo methylation than **3** under alkaline conditions, whereas **3** is methylated smoothly under neutral conditions. This remarkable dependence of the reactivity of **1**, **2**, and **3** on the pH of reaction mixture is useful in view of synthesis, allowing selective methylation of these deoxynucleosides (**1**, **2**, and **3**) by tretament with TMP at a suitable pH. Smooth methylation of **3** at the N-3 position at pH 7 is noteworthy from a biological point of view, since alkylation at the N-3 position of cytosine residues in nucleic acids is believed to be one of the most harmful reactions to result in mutagenicity or carcinogenicity on mice,[14] phage T4V[15] *etc.* Apparently, a Watson-Crick type of base pairing between cytosine bases and guanine bases is no longer possible by methylation of the former at the N-3 position.

Experimental

Melting points are uncorrected. UV spectra were measured with a Hitachi 3T spectrometer. NMR spectra were recorded on a Hitachi Perkin-Elmer R-20 with a dilute solution in deuterioxide and tetramethylsilane as an outside standard or 3-(trimethylsilyl)propionic acid-d_4 sodium salt as an internal standard. Thin-layer chromatography was performed on silica gel [GF_{254} (type 60), Merck] or aluminum oxide [PF_{254} (type 150), Merck] using a mixture of chloroform and methanol. Column chromatography was carried out using silica gel (Merck, Art. 7734, 70—230 mesh) or aluminum oxide (Merck, Art. 1097).

Commercial thymidine (**1**), deoxyuridine (**2**), and deoxycytidine (**3**) as well as TMP were used without further purification.

Spectroscopic Determination of Yields. A mixture of deoxynucleoside (0.25 mmol) and TMP (1.9 mmol, 3.8 mmol, 7.5 mmol, or 14.3 mmol) in water (5.0 ml) was stirred at 37 or 60 °C at an appropriate pH maintained throughout the reaction by occasional addition of 2 M sodium hydroxide, with an error of about ±pH 0.3. At an appropriate reaction time, 4 μl of reaction mixture was spotted on a thin-layer chromatography plate, which was developed immediately with chloroform–methanol (5 : 1 for the reaction of **1** and **2**, and 5 : 2 for that of **3**).

The reaction mixture of **1** with TMP at pH 7—11 showed two spots (**1** and **4**) on TLC (R_f; **1** : 0.07, **4** : 0.23), and a new spot (R_f; 0.38) at pH 12. This was found to be 3,$O^{5'}$-dimethylthymidine by comparison of UV spectrum and R_f value of authentic sample; UV λ_{max} (H_2O) nm : pH 1, 266.0, pH 7, 267.0, pH 13, 267.0. In the reaction of **2** with TMP at pH 7—11, two spots corresponding to **2** and **5** were observed (R_f; **2** : 0.25, **5** : 0.46). Thin-layer chromatography of the reaction mixture of **3** with TMP at 37 °C showed two spots (**3** and **6**) at pH 7 and three spots (**3**, **6**, and **5**) at pH 10 (R_f; **3** : 0.22, **6** : 0.10, **5** : 0.79).

After each spot on TLC was scraped separately from the plate, the substance on the spot was extracted with 3 ml of water. From the absorbance of the solution, the yield of each product was calculated by means of a procedure similar to that described in a previous paper.[8]

Isolation of Products. *3-Methylthymidine (**4**)*: A mixture of **1** (1.20 g, 5.0 mmol) and TMP (10.5 g, 75.0 mmol) in water (15 ml) was stirred at 60 °C and pH 10. After 12 h, the spot of **1** disappeared on thin-layer chromatography of the reaction mixture. The reaction mixture was then neutralized with concentrated hydrochloric acid. After water had been removed from the reaction mixture, the residue was purified by silica gel column chromatography (2.5×35 cm). Elution with chloroform afforded unchanged TMP, **4** being obtained by subsequent elution with chloroform–methanol (7 : 1). Evaporation of the solvent afforded **4** as viscous liquid which crystallized on the addition of diethyl ether (1.13 g, 89.0%); mp 132—133.5 °C (from water) (when recrystallized compound was dried imperfectly, it melted at 84—86 °C) (lit,[16] 132.5—134.0 °C), NMR (D_2O) δ= 1.88 (3H, d, J=1.2 Hz, 5-C**H**$_3$), 2.33 (2H, complex m, 2′-C**H**$_2$), 3.28 (3H, s, N^3-C**H**$_3$), 3.81 (2H, complex m, 5′-C**H**$_2$), 4.03 (1H, complex m, 4′-C**H**), 4.45 (1H, complex m, 3′-C**H**), 6.31 (1H, t, J=7 Hz, 1′-C**H**), and 7.69 (1H, d, J=1.2 Hz, **H**^{6}); UV λ_{max} (H_2O) nm : pH 1, 265.5, pH 7, 266.0, pH 13, 266.0 (lit,[5] pH 1, 265.0, pH 7, 266.0, pH 13, 267.0).

*3-Methyldeoxyuridine (**5**)*: Compound **3** hydrochloride (0.52 g, 2.0 mmol) and TMP (4.2 g, 30.0 mmol) were stirred in water (20 ml, pH 10, NaOH) at 60 °C for 48 h. The reaction mixture was then neutralized with concentrated hydrochloric acid and extracted with chloroform to remove unchanged TMP. The water layer was concentrated to give a residue, which was then purified by silica gel column chromatography (2×33 cm). Elution with chloroform–methanol (7 : 1) provided **5** (120 mg, 25%); mp 109—110 °C (from ethyl acetate–hexane) (lit,[17] 98—100 °C), NMR (D_2O) δ=2.41 (2H, complex m, 2′-C**H**$_2$), 3.32 (3H, s, -C**H**$_3$), 3.85 (2H, complex m, 5′-C**H**$_2$), 4.10 (1H, complex m, 4′-C**H**), 4.45 (1H, complex m, 3′-C**H**), 6.03 (1H, d, J=8Hz, **H**^{5}), 6.39 (1H, t, J=7 Hz, 1′-C**H**), and 7.97 (1H, d, J=8 Hz, **H**^{6}); UV λ_{max} (H_2O) nm : pH 1, 262.5 (log ε 4.12), pH 7, 262.5 (log ε 4.12), pH 13, 263.0 (log ε 4.13).

*3-Methyldeoxycytidine (**6**)*: A mixture of deoxycytidine hydrochloride (1.30 g, 5.0 mmol) and TMP (18.8 g, 134 mmol) in water (50 ml, pH 7) was stirred for 100 h. Alumina thin-layer chromatography of the reaction mixture developed with methanol showed a faint spot of **3** (R_f : 0.12) and a distinct spot of **6** (R_f : 0.72). The reaction mixture was extracted with chloroform in order to remove unchanged

TMP. The water layer was concentrated to give a residue, which was then purified by alumina column chromatography (2.5×40 cm). Elution with methanol afforded the salt of 3-methyldeoxycytidine with dimethyl hydrogen phosphate. Since the phosphate salt was hygroscopic, it was converted into the hydrochloride salt according to the procedure of Furukawa *et al.*[18] (0.78 g, 57%); mp 250 °C (dec); NMR (D_2O) δ=2.55 (2H, complex m, 2′-C**H**$_2$), 3.67 (3H, s, -C**H**$_3$), 3.95 (2H, complex m, 5′-C**H**$_2$), 4.22 (1H, complex m, 4′-C**H**), 4.60 (1H, complex m, 3′-C**H**), 6.30 (1H, d, J=8Hz, **H**^{5}), 6.49 (1H, t, J=7 Hz, 1′-C**H**), and 8.26 (1H, d, J=8 Hz, **H**^{6}); UV λ_{max} (H_2O) nm: pH 1, 279.0, pH 7, 279.0, pH 13, 269.0. The free form of **6** was obtained by subsequent treatment of the hydrochloride salt with anionic exchange resin (Dowex 1×2, 100—200 mesh, OH form); mp 169—171 °C; Found: C, 49.94; H, 6.24; N, 17.29%. Calcd for $C_{10}H_{15}N_3O_4$: C, 49.78; H, 6.27; N, 17.42%.

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