Methylation of Deoxynucleotides with Trimethyl Phosphate in Aqueous Phase

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Action of trimethyl phosphate on thymidine 5′-phosphate, deoxycytidine 5′-phosphate, deoxyadenosine 5′-phosphate, and deoxyguanosine 5′-phosphate at 37—60 °C, and pH 7—10 gave their methyl esters and base-methylated derivatives. Esterification took place in all deoxyribonucleotides to the almost same extent and its rate was enhanced by the reaction temperature, while being independent of pH in the range 7—10. On the other hand, methylation of the base moieties was affected by both the reaction temperature and pH of the reaction medium. The reactivity order of the four kinds of base moieties w s; at pH 7, cytosine base (N-3)>guanine base (N-7)>adenine base (N-1)≫thymine base (N-3); at pH 10, thymine base (N-3)>guanine base (N-1>N-7)>cytosine base (N-3)>adenine base (N-1).

A wide variety of methylated nucleosides have been found in nucleic acids, especially in the 5′-terminal of mRNA[1] and the loop regions of tTNA.[2] The biological roles of the modified nucleic acid-regions have been discussed with respect to the special structures. On the other hand, factitious alkylation of nucleic acids, especially DNA, by means of alkylating agents has been considered an origin of mutagenic effects since the alkylation may furnish the modified nucleotide units which will no longer be capable of performing the normal biological functions.[3] In this context, alkylations of nucleic acids have been studied using various reagents in order to relate the sites and extents of alkylation with mutagenesis.[4-7] The reactions, however, were conducted in a heterogeneous mixture of water and organic solvents mainly because of water-insoluble and -labile properties of the alkylating agents used. We, therefore, employed recently trimethyl phosphate (TMP) as a water-soluble methylating agent, and estimated the reactivity of nucleic acid-bases and nucleosides.[8-11] This paper deals with the study of an action of TMP toward four principal 5′-deoxyribonucleotides (**1**, **5**, **11**, and **16**). Although these monoalkyl phosphates are not the best models for nucleic acids, it was hoped that the study would furnish information about base-methylation and esterification which would be relevant to the methylation of nucleic acids. The reactivity of four deoxyribonucleotides will be also compared.

Results

The reactions were carried out at 37 and 60 °C by stirring a mixture of deoxynucleotide and TMP in water at an appropriate pH. The yields of the products were determined by a thin-layer chromatography (TLC)-UV method. The products were identified conveniently from the mobilities (R_f) in TLC, and spectral characteristics (UV and NMR), or the comparison of the physical constants with those of authentic samples or literature values. Generally, UV spectra were very useful to determine the sites of methylation in the base-rings. The results are summarized in Tables 1—4.

*Thymidine 5′-Phosphate (**1**).* A treatment of **1** with TMP resulted in the formation of three products

TABLE 1. METHYLATION OF THYMIDINE 5′-PHOSPHATE (**1**, pdT) WITH TRIMETHYL PHOSPHATE (TMP)[a]

Temp °C	pH	Product	R_f[b]	λ_{max}[c]/nm at pH 7	UV-yield/% 12 h	24 h	48 h	72 h
37	7	pdT methyl ester (**2**)	0.22	267.0	8	11	19	25
		3-Methyl-pdT (**3**)	0.39	266.0	0	2	2	4
37	8	**2**			7	12	18	21
		3			4	8	14	17
		3-Methyl-pdT methyl ester (**4**)	0.75	267.0	0	Tr[d]	6	12
37	9	**2**			6	8	10	10
		3			17	23	38	43
		4			3	5	13	21
37	10	**3**			50	73	72	63
		4			7	13	24	33
60	7	**2**			39	53	48	41
		3			6	6	4	2
		4			6	15	38	51
60	10	**3**			42	16	—	—
		4			53	78	—	—

a) Reaction size: pdT (0.05 mmol)–TMP (1.5 mmol)–H_2O (0.5 ml). b) These values were measured on cellulose TLC using 1-propanol–concentrated aqueous ammonia–water (7 : 1 : 2). R_f value of pdT was 0.06. c) UV spectra at pH 1 and pH 13 were identical with literature value. d) Tr refers to a trace yield.

Table 2. Methylation of deoxycytidine 5′-phosphate (**5**, pdC) with trimethyl phosphate (TMP)[a]

Temp °C	pH	Product	R_f[b]	λ_{max}[c] /nm at pH 7	UV-yield/% 24 h	48 h	72 h
37	7	pdC methyl ester (**6**)	0.45	271.0	8	13	17
		3-Methyl-pdC (**7**)	0.16	278.0	11	18	22
		3-Methyl-pdC methyl ester (**8**)	0.66	278.0	0	Tr[d]	6
37	10	**6**			11	19	14
		7			9	13	23
		8			Tr	4	7
60	7	**6**			4	6	5
		7			43	59	62
		8			0	15	22
		3-Methyl-pdU methyl ester[e] (**10**)	0.75	263.0	0	0	3
60	10	**6**			33	17	8
		7			5	Tr	0
		8			19	5	Tr
		3-Methyl-pdU[e] (**9**)	0.22	262.0	5	3	4
		10			24	66	83

a) Reaction size: pdC (0.05 mmol)–TMP (1.5 mmol)–H_2O (0.5 ml). b) These values were measured on cellulose TLC using 1-propanol–concentrated aqueous ammonia–water (7 : 1 : 2). R_f value of pdC was 0.09. c) UV spectra at pH 1 and pH 13 were identical with literature value. d) Tr refers to a trace yield. e) pdU refers to deoxyuridine 5′-phosphate.

Table 3. Methylation of deoxyadenosine 5′-phosphate (**11**, pdA) with trimethyl phosphate (TMP)[a]

Temp °C	pH	Product	R_f[b]	λ_{max}[c] /nm at pH 7	UV-yield/% 24 h	48 h	72 h
37	7	pdA methyl ester (**12**)	0.57	259.0	9	14	—
		N^6-Methyl-pdA (**13**)	0.31	267.0	Tr[d]	5	—
37	10	**12**			11	17	—
		13			0	5	—
		N^6-Methyl-pdA methyl ester (**14**)	0.76	266.0	0	1	—
60	7	**12**			41	43	31
		13			11	8	5
		14			8	22	46
		1-Methyl-pdA methyl ester (**15**)	0.19	258.0	Tr	11	12
60	10	**12**			42	12	10
		13			10	Tr	Tr
		14			31	76	85

a) Reaction size: pdA (0.05 mmol)–TMP (1.5 mmol)–H_2O (0.5 ml). b) These values were measured on cellulose TLC using 1-propanol–concentrated aqueous ammonia–water (7 : 1 : 2). R_f value of pdA was 0.19. c) UV spectra at pH 1 and pH 13 were identical with literature value. d) Tr refers to a trace yield.

(**2**, **3**, and **4**) (Table 1). By comparison of the mobilities in TLC and the UV spectra of the aqueous extracts of the spots with those of authentic samples, they were identified as thymidine 5′-(methyl hydrogenphosphate), 3-methylthymidine 5′-phosphate, and 3-methylthymidine 5′-methyl hydrogenphosphate, respectively. It appeared that TMP did not esterify the dialkyl phosphate (**2** and **4**) to afford the respective 5′-dimethyl phosphates. The N-3 methylation on the thymine ring was accelerated significantly with the increase of the pH value, while the esterification rate was almost independent of the pH (7—10). The increase in the reaction temperature was found to promote both the N-3 methylation and the esterification; *viz.* **1** was converted quantitatively into **4** after 24 h at 60 °C and pH 10.

Deoxycytidine 5′-Phosphate (5). A reaction of **5** with TMP in an aqueous solution afforded five UV-absorbing products (**6**, **7**, **8**, **9**, and **10**), of which **9** and **10** were observed only in the reaction at 60 °C (Table 2). Compound **6** was isolated and identified as deoxycytidine

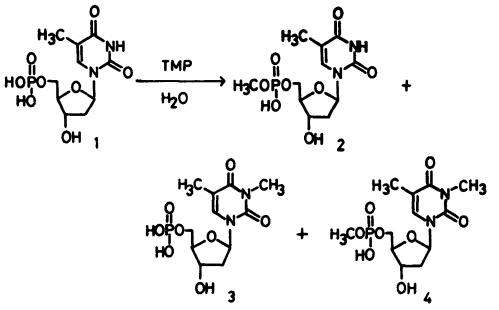


Fig. 1.

TABLE 4. METHYLATION OF DEOXYGUANOSINE 5'-PHOSPHATE (**16**, pdG) WITH TRIMETHYL PHOSPHATE (TMP)[a)]

Temp °C	pH	Product	R_f[b)]	λ_{max}[c)]/nm at pH 7	UV-yield/% 24 h	UV-yield/% 48 h
37	7	pdG methyl ester (**17**)	0.34	252.0 272.0[s)]	6	10
		7-Methyl-pdG (**18**)	0.13	258.0 281.0[s)]	5	6
		7-Methyl- pdG methyl ester (**19**)	0.40	257.0 282.0[s)]	0	2
		7-Methylguanine (**20**)	0.45	284.0	2	6
37	10	1-Methyl-pdG (**21**)	0.14	256.0 269.0[s)]	40	51
		Imidazole ring-opened 7-methyl-pdG (**22**)	0.10	275.0	7	3
		Imidazole ring-opened 1,7-dimethyl-pdG (**23**)	0.18	273.0	Tr[d)]	11
		Unknown A[e)]	0.24	274.0	4	2
		Unknown B[e)]	0.34	258—275	3	11

a) Reaction size: pdG (0.05 mmol)–TMP (1.5 mmol)–H_2O (0.5 ml). b) These values were measured on cellulose TLC using 1-propanol–concentrated aqueous ammonia–water (7 : 1 : 2). R_f value of pdG was 0.06. c) UV spectra of products **17**—**23** at pH 1 and 13 pH were identical with literature value. d) Tr refers to a trace yield. e) The yields of unknown products were determined by postulating their extinction coefficient at λ_{max} as 10000. s) Shoulder.

5'-methyl hydrogenphosphate through its physical properties. Products **7** and **8** were identified tentatively as 3-methyldeoxycytidine 5'-phosphate and its methyl ester, respectively, from their R_f values and UV spectra which were almost similar to those of 3-methyldeoxycytidine. Compounds **9** and **10** were identified as 3-methyldeoxyuridine 5'-phosphate and its methyl ester, respectively, by comparison of their R_f values and UV spectra with those of authentic samples which were prepared from the reaction of deoxyuridine 5'-phosphate with trimethylsulfonium hydroxide.[9)] The compound **9** may be originated from the hydrolysis of the 3-methylcytosine ring of **7**. Similarly, 3-methyldeoxycytidine 5'-(methyl hydrogenphosphate) may be the precursor of **10**, though it may be also produced by the secondary reaction of **9** with TMP.

The esterification of **5** was hardly affected by pH of the solution whereas the N-3 methylation of the cytosine ring was found to decrease slightly with the increase of pH. The increase in the reaction temperature promoted the esterification, the N-3 methylation, and the hydrolysis of **7**, and **8**.

*Deoxyadenosine 5'-Phosphate (**11**).* A treatment of **11** with TMP gave rise to four products (**12**—**15**) as observed in the TLC of the reaction mixture (Table 3). Compounds **12**, **13**, and **14** were identified as deoxyadenosine 5'-(methyl hydrogenphosphate), N^6-methyldeoxyadenosine 5'-phosphate, and N^6-methyldeoxyadenosine 5'-(methyl hydrogenphosphate), respectively, by comparison of R_f values and UV spectra with those of authentic samples. Furthermore, hydrolysis of **12**, and **14** with 1 M hydrochloric acid (1 M=1 mol dm^{-3}) gave adenine and N^6-methyladenine, respectively. Meanwhile, **15** was assigned tentatively 1-methyldeoxyadenosine 5'-(methyl hydrogenphosphate) from the UV spectra (pH 1, 7, and 12) which were very similar to those of 1-methyldeoxyadenosine. As expected, the R_f value was smaller than that of the nucleoside. It would be most likely that the N^6-methylated products (**13**, and **14**) were formed from the Dimroth rearrangement[12)] of the 1-methyl isomers rather than direct methylation at the exocyclic amino groups of the adenine ring, since the external amino group was not as strong in nucleophilicity as the N-1 atom. The esterification, and the N-1 methylation were found to be enhanced by the increase in the reaction temperature, while being almost independent on the pH of the solution.

*Deoxyguanosine 5'-Phosphate (**16**).* Action of TMP on **16** at pH 7 and 37 °C afforded deoxyguanosine 5'-

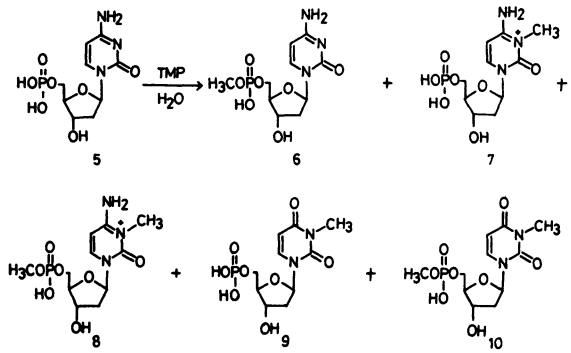


Fig. 2.

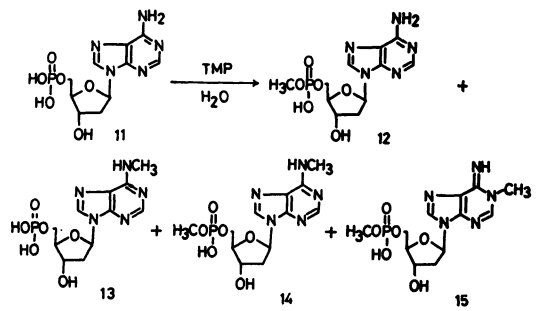


Fig. 3.

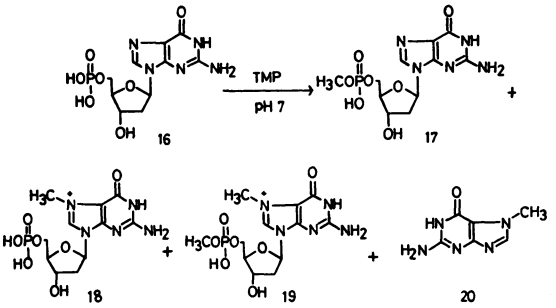


Fig. 4.

(methyl hydrogen phosphate) (**17**), 7-methyldeoxyguanosine 5′-phosphate (**18**), 7-methyldeoxyguanosine 5′-(methyl hydrogenphosphate) (**19**), and 7-methylguanine (**20**).

On the other hand, in the reaction at pH 10 and 37 °C, N-1 methylation took place in preference to both N-7 methylation and esterification, giving chiefly 1-methyldeoxyguanosine 5′-phosphate (**21**). The imidazole ring-opened compounds (**22** and **23**) might be produced by the attack of hydroxide ions at the C-8 position of the 7-methylguanine rings of the corresponding precursors (for instance, **18** for **22**). The reaction mixture also contained two unidentified compounds.

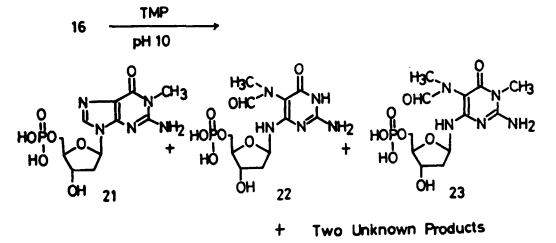


Fig. 5.

All the products other than **22**, and **23** were identified through the comparison of the UV spectra and R_f values with those of authentic samples. Methylation at the N-7 position of the guanine ring was easily acknowledged by the strong fluorescence under the UV light which was characteristic to 7-alkylated guanine ring. The identification of the compounds (**22** and **23**) was tentative, but they had the UV spectra similar to those of the corresponding nucleosides.[11]

Discussion

In a homogeneous aqueous phase TMP was found to methylate the four principal deoxynucleotides at the base moieties (base-methylation) and the phosphate moieties (esterification). As expected, the base methylation proceeded in a manner similar to that of the corresponding nucleosides. Thus, the N-3 of the cytosine ring of **5**, the N-1 of the adenine ring of **11** and the N-7 of the guanine ring of **16** were nucleophilic to be attacked easily by TMP, giving the corresponding products. The pH of the solutions had no effect on methylation at these sites except the N-3 of the cytosine ring which became less susceptive in methylation to the increasing pH. By contrast, the –CO–NH–CO– group of the thymine ring (N^3H) and the –CO–NH– group of the guanine ring (N^1H), which have low pK_a values (9—10), became increasingly active toward methylation with the increasing pH. The reactivity of the base moieties thus can be summarized as follows: pH 7, cytosine (N-3)> guanine (N-7)> adenine (N-1); pH 10, thymine (N-3)> guanine (N-1)> guanine (N-7)> cytosine (N-3).

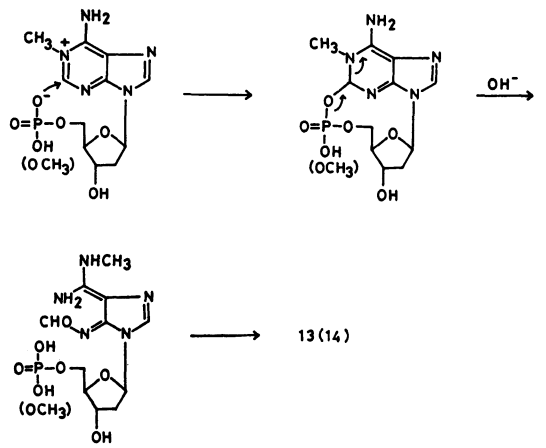
On the other hand, the esterification was hardly affected by the pH but by temperature. Further, variation of the base moieties had no influence on the degree of esterification. Since the nucleotides are monoalkyl phosphates with pK_a of approximately 1 and 6.[13] TMP should attack the dianionic forms of the nucleotides, furnishing the monomethyl ester such as **2**, **6**, **12**, and **17**. Interestingly, the reagent did not methylate further the monomethyl esters to give the dimethyl esters; the results suggest that nucleic acids which are poly(dialkyl phosphates) may not be methylated by TMP at the internucleotide bonds.

Meanwhile, the 5′-phosphate moieties were found to affect the hydrolysis of the 3-methylcytosine ring to the 3-methyluracil ring, and the rearrangement of 1-methyladenine ring to the N^6-methyladenine ring. For instance,

(a) Under the conditions of pH 10, 37 °C, and 24 h, and TMP/substrate=30 mol/mol, 3-methyldeoxycytidine was hydrolyzed more readily than the nucleotides (**7** and **8**); *e.g.* the extent of total N^3-methylation[14] *vs.* that of the hydrolysis was 18% : 2% in the nucleoside,[8] and 17% : 0% in the 5′-nucleotide (Table 2).

(b) Under the conditions of pH 7, 60 °C, and 24 h, deoxyadenosine 5′-phosphate gave predominantly the N^6-methyl derivatives (**13**, and **14**) in the combined yields of *ca.* 19% (Table 3). By contrast, a treatment of deoxyadenosine with TMP under similar neutral conditions produced 1-methyl-, and N^6-methyldeoxyadenosines in the yields of 38%, and trace, respectively.[9]

Plausibly, the phosphate anionic moieties in **7** and **8** were somewhat effective to shield the C-4 positions of the 3-methylcytosine rings from the attack of OH^- ions, thus decreasing the extent of the hydrolysis. On the other hand, the Dimroth rearrangement, which has been considered to occur by the attack of OH^- ions on the C-2 position of the 1-methyladenine ring, might be



Scheme 1.

assisted by the 5′-phosphate moiety as depicted in the Scheme 1. Indeed, adenosine 2′(3′)-phosphate reacted with TMP to give chiefly the 1-methyl derivatives under the conditions (pH 7 and 60 °C) which were the same as those used in the present study.

Dimethyl sulfate, methyl iodide and other S_N2 type reagents have also been reported[4,6,15] to alkylate the deoxynucleotides at the sites similar to those attacked by TMP though the extents of base-alkylation and esterification varied with the reagents used. Highly potent carcinogens, diazomethane and related compounds, have been known to alkylate not only the base nitrogen atoms but also the external oxygen atoms such as thymine ring (O-2 and O-4) and guanine ring (O-6) in addition to the phosphate moieties.[1] The moderate mutagenicity of TMP,[16] thus, may be ascirbed to the predominant methylation at nitrogen atoms, which, except methylation at the N-3 of the cytosine ring, may not disturb significantly the Watson-Crick base pairings.

Experimental

UV spectra were measured with a Hitachi 3T spectrometer. NMR spectra were recorded on a Hitachi Perkin-Elmer R-20 spectrometer, with a dilute solution in deuterioxide and the soldium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d^4 acid as an internal standard. Thin-layer chromatography was performed on cellulose (13254, Eastman) or silica-gel (GF_{254} (type 60), Merck). Column chromatography was carried out using silicagel (Merck, Art. 7734, 70—230 mesh) or aluminium oxide (Merck, Art. 1097).

Commercially available disodium salts of thymidine 5′-phosphate (**1**), deoxyguanosine 5′-phosphate (**16**) and deoxyuridine 5′-phosphate, and the free form of deoxyadenosine 5′-phosphate (**11**) were used without further purification. Deoxycytidine 5′-phosphate (**5**) was prepared according to the procedure of Yoshikawa *et al.*[17] Trimethyl phosphate (TMP) was distilled prior to use.

Reactions of the nucleotides and ethereal diazomethane produced the following known methylated nucleotides,[4,6] which were used to identify the products obtained in the present study: **2**—**4**, **6**, **7**, **9**, **10**, **12**, **15**, **17**—**20**. Some authentic nucleotides were synthesized as follows. They were homogeneous in silica gel- and cellulose-TLC's with the same R_f values as those of known compounds, if any, and had UV spectra and NMR spectra which agreed with the assigned structures.

Thymidine 5′-(Methyl Hydrogenphosphate) (2). The condensation reaction of thymidine 5′-phosphate (**1**, 93 mg, 0.27 mmol) and methanol (0.25 ml) by dicyclohexylcarbodiimde (300 mg, 1.5 mmol) in pyridine (3.0 ml) at 30 °C for 24 h afforded **2**, which was purified through column chromatography and reprecipitation with diethyl ether (60 mg, 66%); NMR (D_2O) $\delta=1.94$ (3H, d, 5-CH_3, $J=1.2$ Hz), 3.63 (3H, d, *p*-OCH_3, $J=11$ Hz), and 7.77 (1H, d, H^6, $J=1.2$ Hz); UV, λ_{max} (H_2O) 267.0 nm.

3-Methylthymidine-5′-(Methyl Hydrogenphosphate) (4). A mixture of **3** (60 mg, 0.2 mmol) and methanolic solution of 0.5 M trimethylsulfonium hydroxide (0.4 ml)[10] was concentrated to give a colorless residue, which was dissolved in DMF (3 ml) and heated at *ca.* 80 °C for 30 min with stirring magnetically. The solvent was evaporated from the reaction mixture, and the resulting residue was applied to a silica-gel column using 1-propanol–concentrated aqueous ammonia–water (7 : 1 : 2 v/v) as a solvent. The title compound was obtained as powder upon mixing the concentrated fraction with diethyl ether, 43 mg (71%), NMR (D_2O) $\delta=1.96$ (3H, d, 5-CH_3, $J=1.2$ Hz), 3.32 (3H, s, N^3-CH_3), 3.63 (3H, d, *p*-OCH_3, $J=11$ Hz), and 7.80 (1H, d, H^6, $J=1.2$ Hz), UV λ_{max} (H_2O) 266.0 nm.

General Methylation Procedure of Deoxyribonucleotide and TMP. A deoxynucleotide (0.05 mmol) was dissolved in water (0.5 ml) of appropriate pH (7—10), and the solution was treated with TMP (1.5 mmol) at 37 or 60 °C. The pH of the reaction mixture was maintained within $\pm$0.5 unit by the occasional addition of 0.5 M sodium hydroxide throughout the reaction. At an appropriate reaction time, 6 μl of reaction mixture was spotted on a cellulose TLC and developed immediately. The product on each spot was scraped from the plate and extracted with 3 ml of water. The yield of each product was calculated by means of a procedure similar to that mentioned in a previous paper,[10] the result of which is summarized in Tables 1—4. The structure of each product was determined mainly by the comparison of its R_f value and UV spectrum with those of the authentic sample.

Methyl ester of deoxycytidine 5′-phosphate (**6**) was isolated from the reaction mixture as follows. After 50-h stirring of a mixture of disodium salt of deoxycytidine 5′-phosphate (**5**, 350 mg, 1.0 mmol) and TMP (4.25 g, 30.0 mmol) in water (10 ml) at 60 °C, pH 7, TMP was removed by extraction with chloroform. The aqueous layer was concentrated to give a residue, which was subjected to alumina column chromatography (1.5×50 cm). Elution with 1-propanol–concentrated aqueous ammonia–water (7 : 1 : 2) afforded **6** (34 mg, 10%); NMR (D_2O) $\delta=3.67$ (3H, d, *p*-OCH_3, $J=11$ Hz), 6.22 (1H, d, H^5, $J=8$ Hz), and 8.07 (1H, d, H^6, $J=8$ Hz),

Products from the reaction mixture of deoxyadenosine 5′-phosphate (**11**) with TMP were further analyzed through the isolation and subsequent acid hydrolysis as follows. Each product in the reaction mixture was separated by silica-gel column (1.5×15 cm) which was eluted with 1-propanol–water (3 : 1). Subsequently, each product was hydrolyzed by 1 M hydrochloric acid at 90 °C for 3 h. Adenine was obtained near quantitatively from **12**, and N^6-methyladenine from **13** and **14**, and 1-methyladenine from **15**. The products were identified by UV spectra and comparison of R_f values with those of the authentic sample using several solvent systems.

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