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The study of far UV irradiation of 2-methoxycytosine in phosphate solution and its novel photoproduct

Feng Lin *, Jin-long Wu, Jia-geng Liu

Department of Chemistry, Zhejiang University, Hangzhou 310028, China

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Abstract

The reaction occurring when 2-methoxycytosine(2-methoxy-4-aminopyrimidine, MAP) is irradiated by a middle pressure mercury lamp (MPML) in aqueous phosphate buffer at pH 8 has been studied. We have found that the course of photoreaction of MAP is strongly affected by the presence of phosphate, and some similar to the experimental phenomenon of Moore [Moore, A.M., (1963) Can. J. Chem., 41, 1937–1956], who studied that MAP was irradiated by a low pressure mercury lamp (LMLP) in aqueous phosphate buffer at pH 7, and indicated that the product isolatable in greatest yield is N-carbomethoxy-3-hydroxyacylamidine (**I**). But in our experimental the product of highest yield, isolated after irradiation of MAP, is 2-methoxy-6-phosphatecytosine (**II**), and no **I**. Experimental results indicated that the UV irradiation (190–220 nm) in the emission spectra of MPML is responsible for the phosphate-dependent photoreaction of MAP. Phosphates absorbing the UV (190–220 nm) energy are converted to phosphate anion radicals, which react with MAP in the presence of oxygen, leading to a novel compound **II** with phosphate group ($C_5H_8N_3O_5P$). The composition and structure of the compound has been identified by elemental analysis, EI-MS, UV, IR, ^{1}H, ^{13}C and ^{31}P-NMR. The photochemical behaviors of MAP in the presence of phosphate have been discussed. ©2000 Elsevier Science S.A. All rights reserved.

Keywords: UV irradiation; 2-methoxycytosine; Photoproduct

1. Introduction

The photolysis of 2-methoxycytosine (MAP) in phosphate-buffer solution under low pressure mercury lamp (LPML, 254 nm) was reported as early as 1963 by Moore [1], who found that the presence of inorganic orthophosphate altered the course of the photolysis process of MAP (phosphate effect), and produced an new compound **I** (Scheme 1). He studied the effect of phosphate concentration and pH on the formation of I and concluded phosphate acted a 'catalyst' in the photolysis of MAP by some unknown reaction mechanism.

Phitha and Butler [2] extended Moore's work and studied the effect of phosphate buffer on the photoreactions of a number of pyrimidines. They found that the presence of phosphate effect had in only four of the compounds studied. These substances had in common a methyl or a methoxy group in 2-position and an amino group at the 4-position. It was suggested that these features are important in determining a pyrimidine susceptible to the phosphate effect.

Szabo [3] and Shaw [4] confirmed the experimental result of Moore, but the reaction mechanism had not been elucidated. In particular, the role of phosphate in the mechanism was not clear.

In the study on the damage of UV-induced DNA, although the ionization or another photoreaction of the sugar-phosphate has been proposed as a possible explanation for the higher quantum yield for frank strand breaks at 193 and 199.8 nm [5], previous investigations both in photochemistry and in radiochemistry of nucleic acids tend to deny the role of phosphate in the photolysis of nucleobases, nucleosides and nucleotides (NA). Until recently it has still been claimed that photoionized phosphate groups of the sugar-phosphate moiety by 193 nm UV quanta are not major precursors to the single strand breaks of DNA [6,7].

In a previous paper [8,9] we found that the photoreactive loss of NA was sharply enhanced by phosphate undergoing MPML irradiation (continuous spectrum) and got a novel photoproduct (6-phosphatecytosine) in the photoreactive system of cytosine-phosphate solution. Now we have studied the photoreaction of MAP in phosphate solution

* Corresponding author. Tel.: +86-0571-8076539; fax: +86-0571-5376772.
E-mail address: linfeng@mail.hz.zj.cn (F. Lin).

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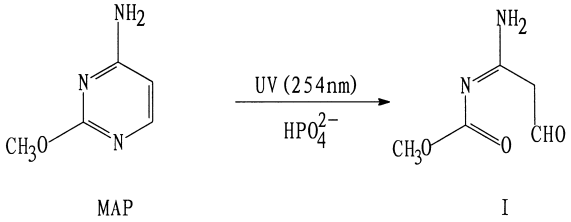
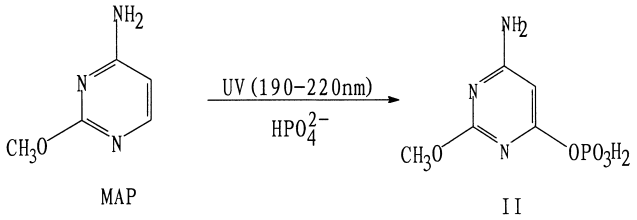


Scheme 1.



Scheme 2.

at pH 8 by irradiation of MPML and found the photoreactive enhancement of MAP by added phosphate, which is similar to Moore's phosphate effect, and but produce a novel compound II with phosphate group (Scheme 2) that is different from Moore's photoproduct. Filter light experiment indicated that the UV irradiation (190–220 nm) energy in the emission spectra of MPML is responsible for the phosphate-dependent photoreaction of MAP. It is suggested that the photoreaction mechanisms of MAP in phosphate solution are different under irradiation between MPML and LPML.

2. Experimental

2.1. Chemicals

2-Methoxycytosine (MAP) was synthesized according to Hilbert and Johnson [10]. The structure and purity of synthesized MAP was checked by proton NMR and elemental analysis. Phosphate salts (KH_2PO_4, $K_2HPO_4{\cdot}3H_2O$) were supplied by Hangzhou Chemical Reagent Factory and recrystallized in hot water before use. Triply distilled water was used to prepare all the solutions.

2.2. Far UV irradiation

The irradiation equipment was self-built. A 400 w MPML was employed as irradiation source [11]. Phosphate buffer saline (PBS. pH 8) was used both as a phosphate source and at the same time as a buffer to minimize the influence of pH changes on the photoreactive loss of MAP. Typically, photoreaction was done in air-saturated solution of 1×10^{-4} mol/dm^3 MAP and 0.05 mol/dm^3 phosphate buffer at pH 8, which was irradiated in a 1 cm length quartz cell (Beckman) that was placed 20 cm away from MPML to keep the temperature of solutions in cell at about 30°C. Deoxygenation was carried out by bubbling 99.99% pure nitrogen through the solution stored in a special quartz tube ($\phi1\times5$ cm) for 30 min when necessary.

2.3. Preparation and isolation of photoproduct

Preparative solutions were directly irradiated in glass vessel surrounded by ice-water to keep the temperature <5°C and well mixed with a magnetic stirrer. Typically, 200 ml solution of 1×10^{-4} mol/dm^3 MAP and 0.05 mol/dm^3 phosphates at pH 8 was irradiated for 1 h, and then chromatographed on an anion exchange column of Zeolit FF (200–400 mesh). The column was washed with 1 litre of 0.1 mol/dm^3 formic acid at the rate of 1 ml/min. The eluent was collected in 10 ml fractions. A sample of each fraction was analyzed by ultraviolet spectrophotometer immediately after it emerged from the column. The total eluents containing photoproducts were combined and evaporated to dryness on a rotary evaporator. The photoproduct was further purified by recrystallization in water.

2.4. Filter UV light experiment

The MPML emits a continuum possibly covering the range from less than 190 nm to visible light. The UV quanta less than 190 nm can be filtered by 1 cm length water filter which is placed before the irradiation solution. The UV quanta less than 220 nm can be filtered by 1 cm length of 20% acetic acid solution [11].

2.5. The photoreactive loss of MAP and the yield of phototproduct

The photochemical behaviors of MAP in the presence of PBS are characterized by the photoreactive loss of MAP. It is defined as follows:

$$\text{The photoreactive loss of MAP (\%)} = \frac{[\text{MAP}]_{\text{unirradiated}} - [\text{MAP}]_{\text{irradiated}}}{[\text{MAP}]_{\text{unirradiated}}} \times 100$$

$$\text{The yield of photoreactive (\%)} = \frac{[\text{photoproduct}]}{[\text{MAP}]_{\text{unirradiated}}} \times 100$$

The concentration of MAP and photoproduct is analyzed by high-performance liquid chromatography(HPLC) on a ϕ 4.6×250 mm Zorbax SB C18 column with Varian Model 5060 HPLC-Vista-401 Data System. The sample was injected into the column and eluted with triply water at a flow rate of 1 ml/min. The UV detector was set at 270 nm.

3. Results and discussion

Under UV irradiation of MPML the photoreaction of MAP was sharply enhanced by phosphates. The far-UV absorption spectra of KH_2PO_4, K_2HPO_4, Na_2HPO_4 on aqueous media are composed of both very weak bands from 300 nm to 220 nm and steep absorption edges below 220 nm [12]. The former region is assigned to $n\rightarrow\pi^*$ transition, while the intense absorption band below 220 nm is due to a charge-transfer-to solvent type transition [13]. Phosphates can absorb the energy of the UV(190–220 nm), and convert to phosphate anion radicals, which react with MAP and enhance the photoreaction of MAP, which is different from the photochemical behavior under the irradiation of LPML [1].

3.1. Photochemical behaviors of MAP in the presence of phosphate under the irradiation of MPML

3.1.1. The photoreaative loss of MAP and the yield of photoproduct as the function of the irradiation time

Fig. 1 reveals that under UV irradiation the photoreactive loss of MAP-PBS is evidently higher than that of pure MAP solution, that is, PBS can enhance the photoreaction of MAP. But the enhancement effect of PBS will be eliminated if using 20% acetic acid solution as a filter, which absorbs the UV light (<220 nm) completely. As shown in Fig. 2 the yield of photoproduct at first increase to the irradiation time in the range of 30 min, and then drops slowly due to the competition reaction of photoproducts. In order to minimize the influence on the photolytic yield of the secondary reactions during the irradiation of MAP, the irradiation time was controlled in 10 min.

3.1.2. Dependence of the enhancement and the yield of photoproduct on concentration of MAP and K_2HPO_4

The dependence of the enhancement on MAP concentration is presumed due to the competitive absorption of the incident photons between MAP and Phosphates. Although

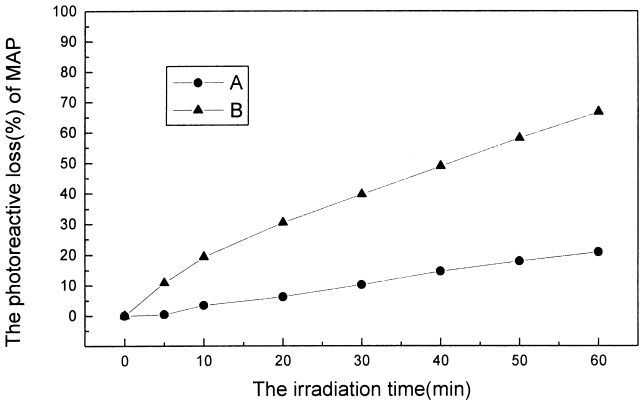


Fig. 1. The photoreactive loss of MAP as the function of the irradiation time [MAP]=1.00×10^{-4} mol/dm^3, pH=8, A=no phosphate, B=0.05 mol/dm^3 phosphate.

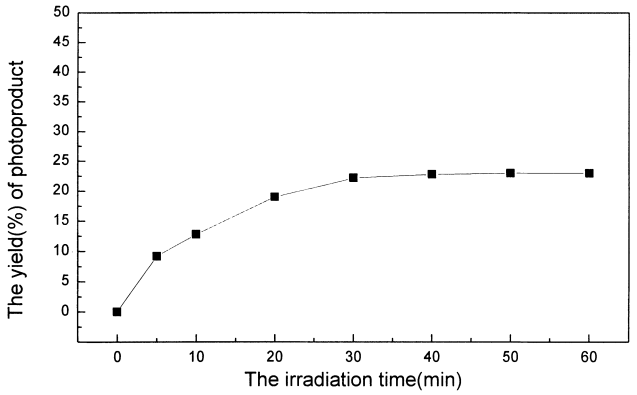


Fig. 2. The yield of photoproduct as the function of the irradiation time [MAP]=1.00×10^{-4} mol/dm^3, pH=8, [k_2HPO_4]=0.05 mol/dm^3.

the molar extinction coefficient of PBS at 193 nm is about 100-fold less than that of MAP [1,12], the concentration of PBS (5.00×10^{-2} mol/dm^3) is much higher than that of MAP (1.00×10^{-4} mol/dm^3). It can be estimated approximately that 80% of UV light (near 200 nm) will be absorbed by PBS in this photoreactive system. But the photoreactive loss of MAP was sharply decreased with the increase on the concentration of MAP by reason that the incident UV light (190–220 nm) is mainly absorbed by MAP (Fig. 3A) and, at this condition PBS can not form phosphate anion radicals. Furthermore, the yield change of photoproduct with the concentration of MAP less than 1.00×10^{-4} mol/dm^3 was explained due to the same reasons (Fig. 3B).

The photoreactive loss and the yield of photoproduct increased on the increasing of PBS concentration in the range of less than 5.00×10^{-2} mol/dm^3 (Fig. 4). The slight decrease after the maximum yield of photoproduct with further increase of PBS concentration is presumed to the self-termination of the phosphate radicals rather than their interaction with MAP (Fig. 4B).

3.1.3. The photoreactive loss of MAP and the yield of photoproduct as the function of pH

The photochemical behavior of the system is rather complex due to the dissociation equilibrium of MAP as function

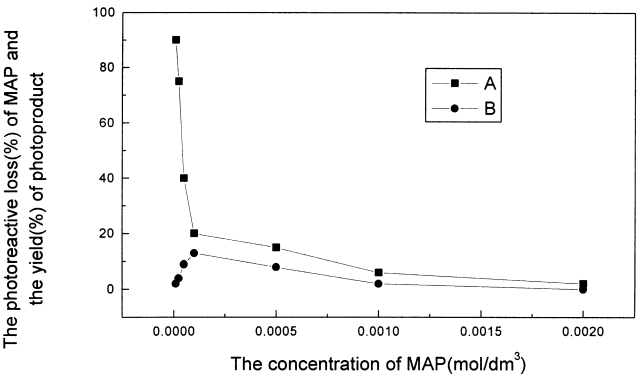


Fig. 3. The photoreactive loss(A) of MAP and the yield(B) of photoproduct as the function of the concentration of MAP in 0.05 mol/dm$_3$ k_2HPO_4 (pH=8).

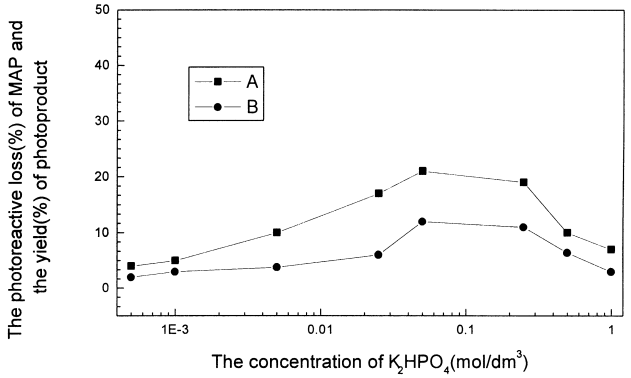


Fig. 4. The photoreactive loss(A) of MAP and the yield(B) of photoproduct as the function of the concentration of K_2HPO_4 in 1.00×10^{-4} mol/dm^3 MAP.

of pH (Fig. 5A) and the phosphate anions are known to undergo proton dissociation with increase in pH [14].

Although the MAP-PBS photoreactive solution at pH 2–3 had the higher photoreactive loss, the photoproduct found at pH 8 was not detected by HPLC. The yield of photoproduct is strongly dependent upon the pH of solution, which at pH 8 reaches the highest yield of product and at less than pH 5 no the photoproduct (Fig. 5B). It illustrated that MAP exists as a neutral molecule form and is beneficial in producing the novel compound **II**. In order to obtain a similar effect of the phosphate radicals with MAP, all the experiments were performed at pH 8.

3.1.4. Photoproduct Identification of MAP

3.1.4.1. Elemental analysis. $C_5H_8N_3O_5P$ Calc.: C: 27.15%, H: 3.62%, N: 19.01%, P: 14.02%, O: 36.20%, Found: C: 26.92%, H: 3.86%, N: 19.22%, P: 13.85%, O: 36.60% (by difference).

3.1.4.2. Ultraviolet absorption spectroscopy. The UV absorption peak of an aqueous solution of MAP at pH 7.0 is at 268 nm. But the ultraviolet spectrum of photoproduct at pH 7.0 exhibited two maxim at 274 nm and 208 nm, with the molar extinction coefficient 6.56×10^3 and 2.23×10^4 dm^3·mol^{-1}·cm^{-1}, respectively. The absorption peak at 208 nm is assigned to –P=O bond excitation. The absorption peak at 274 nm with higher molar extinction coefficient reveals that it has a conjugate system.

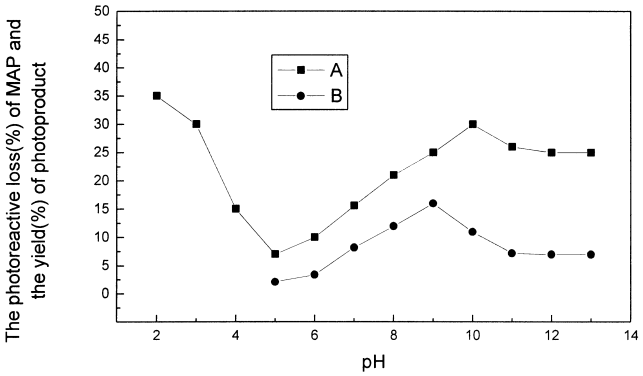


Fig. 5. The photoreactive loss(A) of MAP and The yield(B) of photoproduct as the function of the pH in 1.00×10^{-4} mol/dm^3 MAP and 0.05 mol/dm^3 k_2HPO_4.

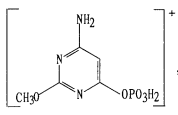
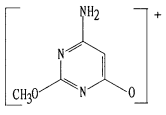
Table 1
IR data of the functional groups and bands in the photoproduct

Vibration frequency (cm^{-1})	Functional groups and bonds
3420 3219	$–NH_2$, –C–NH_2, N–H and $–NH_3^+$ stretching
1640	–C=C– stretching; $–NH_2$ shear stretching
1514	C=N stretching
1249	P=O stretching
1163 1122	P–O–C stretching
996 960	P–O–H stretching

3.1.4.3. Infrared absorption spectroscopy. IR data of the functional groups and bands of the photoproduct are shown in Table 1.

The infrared absorption spectroscopy appeared to be consistent with supposed structure.

3.1.4.4. Mass spectrometry. The ES-MS of photoproduct was carried at Model ZAB organic mass spectrometer. The mass and relative amount of the ionized fragments of the photoproduct molecule were measured. The major peak appeared at mass 221(22), 139(100), 165(42), 84(21), 31(28). On comparing the possible atomic composition of these fragments with the proposed structure. It appeared probably that: mass 165 represents: $[HN{=}CNH_2–CH{=}C–OPO_3H_2]^{+\cdot}$,

mass 221 represents:  , mass 139 represents: 

mass 84 represents: $[HN{=}CNH_2–CH{=}C–O]^{+\cdot}$, mass 31 represents: $[CH_3O]^+$. These fragments appeared to be consistent with the supposed structure.

3.1.5. Nuclear magnetic resonance spectroscopy

^{1}H-NMR spectroscopy (500 MHz, $CDCl_3$): δ_{ppm}: 3.82 (s, 3H); 7.81 (d, J=5 1H).

^{31}P-NMR spectroscopy (85% H_3PO_4 as external standard, δ_{ppm}=0.77 ppm): δ_{ppm}: 0.81.

It meant the chemical surrounding of P in the photoproduct was similar to that of H_3PO_4 .

^{13}C-NMR Spectroscopy: The ^{13}C-NMR spectrum data photoproduct are listed in Table 2.

C(6) and C(5) split into two peaks caused by the contention of $–OPO_3H_2$ group coupling.

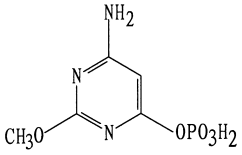
Table 2
^{13}C-NMR data of the photoproduct and parent MAP

$\delta_{C(ppm)}$	C_2	C_4	C_5	C_6
Photoproduct	135.64	148.12	82.91	126.85
MAP	145.21	158.30	91.95	138.20

Table 3
The effect of O_2 and N_2 in photoreactive system

Condition	The photoreactive loss (%) of MAP	The yield (%) of photoproduct
Air saturated	22.0	12.0
O_2 saturated	31.0	17.5
N_2 saturated	3.50	0

From the identification results, it can be confirmed that the isolated photoproduct is 2-methoxy-6-phosphatecytosine, its proposed structure is as follows:



3.1.6. Formation mechanism of photoproduct

Since all irradiation were carried out under conditions such that 80% of the light (190–220 nm) was absorbed by the phosphate dianions, the initial process must be a transition of HPO_4^{2-} to electronically excited states $(HPO_4^{2-})^*$, then released an electron to the water solvent [12,13] and produced phosphate anion radicals (HPO_4^-). The formation mechanism of 2-methoxy-6-phosphate cytosine in the photoreaction of MAP in PBS has not elucidated, although the result is clearly the addition of HPO_4^{2-} at the C6 position of MAP. According to the results of Table 3 and the comparison of the photoreactive loss of MAP and the yield of photoproduct, oxygen is necessary for the formation of the photoproduct.

Behrens, G., et al. [15] studied the addition of phosphate dianions to the photolysis solutions of persulphate and pyrimidine bases, which yielded the ESR spectra of the C(6)-phosphate radicals , but they had not obtained the product.

4. Conclusions

1. The photoreaction of MAP was sharply enhanced by PBS under the irradiation of MPML, but the photoreactive mechanism is not alike to the irradiation of LPML.
2. The UV irradiation (190–220 nm) in the emission spectra of MPML is responsible for the phosphate-dependent photoreaction of MAP . The main photoproduct from the photoreaction of MAP-PBS system in the presence of O_2 is 2-methoxy-6-phosphatecytosine.
3. The yield of photoproduct strongly varied with the pH of solution. If in acid solution (at less than pH 5), the photoreactive system of MAP-PBS will not produce the novel photoproduct found at pH 8.

It suggested that the reacting species are the unionized amino form of MAP and phosphate anion radicals (HPO_4^-).

Acknowledgements

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References

[1] A.M. Moore, Can. J. Chem. 41 (1963) 1937.
[2] P.M. Pitha, G.C. Butler, Can. J. Biochem. 46 (1968) 893.
[3] A.G. Szabo, K.B. Berens, Photochem. Photobiol. 21 (1975) 141.
[4] A.A. Shaw, M.D. Sheltar, Photochem. Photobiol. 49 (1989) 273.
[5] I.G. Gut, R.C. Farmer, C. Huang, I.E. Kochevar, Photochem. Photobiol. 58 (1993) 313.
[6] L.P. Candeias, S. Steenken, J. Am. Chem. Soc. 114 (1992) 699.
[7] T. Melvin, M.A. Plumb, S.W. Botchway, Photochem. Photobiol. 61 (1995) 584.
[8] F. Lin, W.-Q. Wang, J.-L. Wu, Chem. Res. Chin. Univ. 14 (1998) 365.
[9] H.-X. Li, W.-Q. Wang, F. Lin, J.-L. Wu, Bio. Sci. Space. 12 (1998) 160.
[10] G.E. Hilbert, T.B. Johnson, J Am. Chem. Soc. 52 (1930) 1152.
[11] K.-J. Wang, X.-M. Pan, W.-Q. Wang, Photochem. Photobiol. 65 (1997) 656.
[12] Halmann, M., Platzner, I., J. Chem. Soc. (1965), 1440.
[13] M. Halmann, I. Platzner, J. Phys. Chem. 70 (1966) 2281.
[14] E.D. Black, E. Hayon, J. Phy. Chem. 74 (1970) 3109.
[15] Behrens, G., Hildenband, K., et al., J. Chem. Soc. Perkin Trans II. (1988), 305.