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DNA cleavage induced by photoirradiation of coenzyme B_{12} and organocobaloximes without dioxygen

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Abstract

Homolytic cobalt–carbon bond cleavages of methylcobalamin (coenzyme B_{12}) and a B_{12} analogue, $[(DH)_2(CH_3)Co(py)]$ ($(DH)_2$ = bis(dimethylglyoximate), py = pyridine) by UVA irradiation induced effective DNA strand scission by generated methyl radical under anaerobic conditions. The efficiency of DNA strand scission is drastically changed depending on the type of generated radicals. For example, the photoexcitation of 5′-deoxyladenosylcobalamin (one of coenzyme B_{12}) under anaerobic condition results in less effective DNA cleavage. No DNA cleavage has occurred under photoirradiation of $(DH)_2(PhCH_2)Co(py)$, because the reactivity of benzyl radical formed by Co–C bond cleavage is much lower than that of methyl radical. The photoreactivity of coenzyme B_{12} and organocobaloximes toward DNA nucleotides was also examined in order to compare the reactivity with those of DNA cleavage. In contrast to the efficient DNA strand scission by photoirradiation of methylcobalamin and $(DH)_2(CH_3)Co(py)$ in the absence of oxygen, the photoirradiation in the presence of oxygen under otherwise the same experimental conditions resulted in no DNA cleavage. This indicates that the DNA cleavage by alkyl radicals is suppressed by molecular oxygen, because alky radicals are converted to the much less reactive alkylperoxyl radicals.

Keywords: DNA; Coenzyme B_{12}; Radical; Oxygen; Photoirradiation

1. Introduction

DNA damage is well known to play a critical role in many diseases [1–5]. The oxidative DNA damage has been extensively studied by using reactive oxygen species (ROS) [1,6–8]. On the other hand, the formation of methyl radical derived from photo-induced metal–carbon bond cleavage of organometallic complexes that bind to DNA is also reported to induce DNA cleavage by the attack of methyl [9] or methoxyl radical [10]. In contrast to the oxidative DNA damage, however, relatively little is known for DNA damage by alkyl radicals in spite of the importance of the radical activity in living systems [1].

Coenzyme B_{12}, which plays an important role in a number of enzymatic reactions [11–17], is known to induce the homolytic cobalt–carbon bond cleavage that results in formation of the corresponding alkyl radical ($R^{\bullet}$) and Co(II) species under enzymatic reaction conditions or photoirradiation [18]. There have been a number of reports that demonstrate photoinitiated strand cleavage of DNA by a wide variety of transition metal complexes [19–22]. As for cobalt complexes, photoirradiation of Co(III) bleomycin (BLM) is well known to induce DNA strand cleavage [23,24]. The DNA cleavage mechanism was proposed that HOO–Co(III) BLM, produced by the photoirradiation in the presence of oxygen, initiated DNA strand scission through C4′–H abstraction resulting in site selective damage [24]. However, the direct DNA cleavage by radicals generated by photoirradiation of coenzyme B_{12} or B_{12} analogues has yet to be reported.

We report herein that photo-induced homolytic cobalt–carbon bond cleavage of coenzyme B_{12} and organocobaloximes (B_{12} analogues) results in effective DNA strand scission under anaerobic conditions depending on the type of produced radicals. The photoreactivity of coenzyme B_{12} and organocobaloximes toward DNA nucleotides was also examined in order to compare the reactivity with those of DNA cleavage. The effect of oxygen on the photo induced DNA cleavage by coenzyme B_{12} and organocobaloximes is also reported.

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2. Experimental

2.1. Materials

Methylcobalamin (CH_3Cbl) and adenosylcobalamin (AdoCbl) were purchased from Sigma Chemical Co. Alkylcobalt(III) complexes, [$(DH)_2$(R)Co(py)] (R = CH_3 and $PhCH_2$, $(DH)_2$ = bis(dimethylglyoximate), py = pyridine) were prepared by following the literature method [25]. DNA pBR322 (0.24 μg μL^{-1}) were purchased from Wako Pure Chemical Ind. Ltd., Japan. Nucleotides, GMP (guanosine 5′-monophosphate) and AMP (adenosine 5′-monophosphate) were obtained from Nacalai Tesque, Japan and, TMP (thymidine 5′-monophosphate) and CMP (cytosine 5′-monophosphate) were obtained from Aldrich. Calf-thymus deoxyribonucleic acid, sodium salt (DNA) was purchased from Sigma Chem. Co., USA. Potassium ferrioxalate used as an actinometer was prepared according to the literature and purified by recrystallization from hot water [26]. Purification of water (18.3 MΩ cm) was performed with a Milli-Q system (Millipore; Milli-Q Jr.). Acetonitrile (CH_3CN) and propionitrile used as solvent were purified and dried by the standard procedure [27].

2.2. DNA cleavage

A 2 μL of aqueous solution of DNA pBR322 (0.24 μg μL^{-1}) was dried *in vacuo*. Typically, 20 μL of aqueous buffer solution of coenzyme B_{12} or 20 μL of aqueous buffer solution containing 2 μL of CH_3CN of organocobaloxime and dried DNA were mixed in a microtest tube. A deaerated aqueous buffer solution was prepared after three freeze-pump-thaw cycles. Samples were incubated under irradiation with a monochromatized light (λ_{max} = 340, 370, or 520 nm) from a Shimadzu RF-5300PC spectrophotometer at 298 K. The 2 μL of aqueous solution of DNA pBR322 was diluted by adding 18 μL of water, then mixed with 2 μL of loading buffer (0.1% bromophenol blue and 3.75% Ficol in TAE buffer) and loaded onto 1.4% agarose gel. The gel was run at a constant voltage of 130 V for 50 min in TAE buffer using a Nihon Eido electrophoresis kit, then washed with distilled water, soaked into 0.1% ethidium bromide aqueous solution, visualized under a UV transilluminator, and photographed using a digital camera.

2.3. ESR measurements

In an experiment of the ESR measurements for the detection of Co(II) species, 0.4 mL of a deaerated aqueous buffer solution (5 mM Tris–HCl (pH 7.0)) of CH_3Cbl (1.6×10^{-3} M) and TMP (2.3×10^{-1} M) was in an ESR sample tube (internal diameter: 4 mm). In experiments of the ESR measurements for the detection of Co(II) species under aerobic conditions, 0.4 mL of an O_2-saturated aqueous buffer solution (5 mM Tris–HCl (pH 7.0)) of CH_3Cbl (1.8×10^{-3} M) or an O_2-saturated propionitrile solution containing $(DH)_2(CH_3)Co(py)$ (2.9×10^{-2} M) was in an ESR sample tube. The ESR sample was then irradiated with a 1000-W high-pressure mercury lamp (Ushio-USH1005D) through an aqueous filter at 298 K, then immediately frozen and measured at 77 K. ESR spectra were measured with a JEOL JES-ME-LX and were recorded under nonsaturating microwave power conditions. The magnitude of the modulation was chosen to optimize the resolution and the signal-to-noise ratio (S/N) of the observed spectra. The *g*-values were calibrated using an Mn^{2+} marker.

2.4. Quantum yield determination

A standard actinometer (potassium ferrioxalate) [26] was used for the quantum yield determination of the photochemical reactions. A square quartz cuvette which contained a 5 mM Tris–HCl buffer solution (pH 7.0) of CH_3Cbl and nucleotides was irradiated with monochromatized light of λ_{max} = 340 nm from a Shimadzu RF-5300PC fluorescence spectrophotometer. Under the conditions of actinometry experiments, both the actinometer and CH_3Cbl absorbed essentially all the incident light. The light intensity of monochromatized light of λ_{max} = 340 nm was determined as 1.6×10^{-8} einstein s^{-1}. The photochemical reaction was monitored using a Shimadzu UV-3100PC spectrophotometer. The quantum yields were determined from the decrease from the absorbance due to CH_3Cbl (λ = 520 nm, $\varepsilon = 7.6 \times 10^3$ M^{-1} cm^{-1}).

2.5. Product analysis

After completion of the photoreaction of CH_3Cbl (8.0×10^{-5} M) with nucleotides (0.4 M) or DNA (1.2×10^{-2} M) in a square quartz cuvette sealed with rubber septum, the gaseous products were analyzed by GC using an Active Carbon column. A commercial standard gas (CH_4, 99.7%) from GL Science Co. Ltd., Japan, was used as a reference. The determination of the yield was made using sample cuvette of the same size after equilibrium of the reference gas.

3. Results and discussion

3.1. Photo induced DNA cleavage by coenzyme B_{12} and analogues

DNA-cleavage reactivity by alkyl radicals generated under photoirradiation of coenzyme B_{12} and organocobaloximes (Fig. 1) was examined using the widely handled assay with pBR322 supercoiled DNA by agarose gel electrophoresis [7,28].

Photoirradiation of an aqueous DNA solution containing methylcobalamin (CH_3Cbl), one of coenzyme B_{12}, and organocobaloxime, $(DH)_2(CH_3)Co(py)$ under N_2 results in effective cleavage of plasmid DNA (supercoiled form I) to afford the nicked form II as shown in Fig. 2a and d. The monochromatized visible light irradiation of the absorption band of coenzyme B_{12} (λ_{max} = 520 nm) also results in effective DNA cleavage as shown in Fig. 2e. Photoexcitation of 5′-deoxyladenosylcobalamin (AdoCbl) under N_2, which is one of coenzyme B_{12}, results in less effective DNA cleavage as shown in Fig. 2c. In contrast to the case of $(DH)_2(CH_3)Co(py)$, no cleavage of plasmid DNA has occurred under photoirradiation

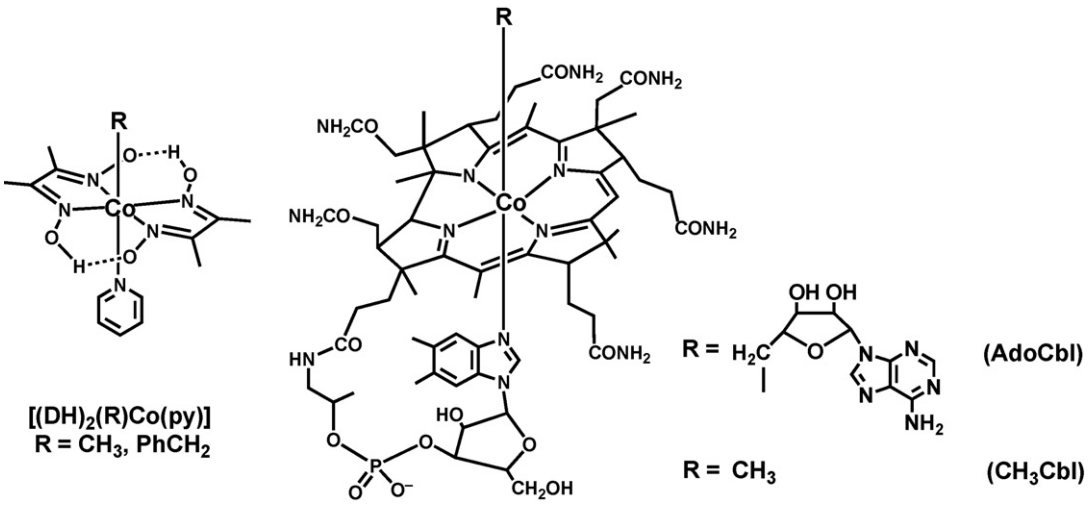


Fig. 1. Structures of organocobaloximes ($(DH)_2(R)Co(py)$) and coenzyme B_{12}.

by $(DH)_2(PhCH_2)Co(py)$ under N_2 (Fig. 2b). To our surprise, the photoirradiation of each system under aerobic conditions results in no DNA cleavage as shown in Fig. 2a–d.

Above-mentioned results strongly implicate high reactivity of methyl radicals for DNA cleavage. However, the presence of oxygen in this system strongly inhibits the DNA cleavage. The reason for this is discussed later.

3.2. Photochemical reactions of coenzyme B_{12} and analogues with DNA nucleotides

The photoreactivity of coenzyme B_{12} and organocobaloximes toward DNA nucleotides was also examined in order to compare the reactivity with those of DNA cleavage. Irradiation of the absorption band of CH_3Cbl ($\lambda = 340$ nm, 8.4×10^{-5} M) in a deaerated aqueous buffer solution (pH 7.0) containing DNA bases resulted in a decrease in the absorbance of CH_3Cbl accompanied by an increase in the absorbance due to the formation of Cob(II)alamin as shown in Fig. 3 ($\lambda_{max} = 474$ nm with clear isosbestic points). Without nucleotides, no photochemical reaction of CH_3Cbl occurred, because the decrease of the absorbance band due to CH_3Cbl was negligible under otherwise the same experimental conditions. This indicates that photocleaved methyl radical reacts nucleotides in competition with the recombination between methyl radical and Cob(II)alamin. The rate of photolysis of CH_3Cbl is also accelerated by oxygen to form aquacobalamin and hydroxocobalamin [29].

The quantum yields (Φ) of the photoreactions of CH_3Cbl with all types of DNA nucleotides (GMP, AMP, CMP, and TMP) in a deaerated aqueous buffer solution (5 mM Tris–HCl (pH 7.0)) were determined from the initial rate of the decay of the absorbance due to CH_3Cbl under photoirradiation of monochromatized light ($\lambda_{max} = 340$ nm). A standard actinometer (potassium ferrioxalate) was used for the quantum yield determination. The Φ values increase with increasing concentrations of DNA nucleotides to approach limiting values (Fig. 4). The reaction mechanism can be represented as shown in Scheme 1, from which the dependence of Φ on concentrations of DNA nucleotides (XMP) can be derived as given by Eq. (1),

$$\Phi = \frac{\alpha k_P[XMP]}{k_R + k_P[XMP]} \quad (1)$$

where α is the quantum yield for the cobalt–carbon bond cleavage of CH_3Cbl, k_R and k_P are the rate constants of the geminate recombination between $CH_3^\bullet$ and Cob(II)alamin in the cage and the reactions of $CH_3^\bullet$ with XMP, respectively. According to Scheme 1, the rate equations are given by Eqs. (2) and (3),

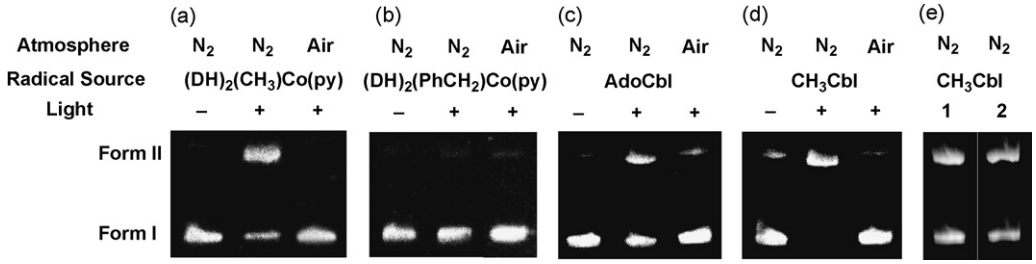


Fig. 2. Agarose gel electrophoreses of cleavage of supercoiled pBR322 DNA (3.7×10^{-5} M) in an aqueous buffer solution (10 mM KH_2PO_4/NaOH, pH 7.0) containing: (a) $(DH)_2(CH_3)Co(py)$ (4.4×10^{-3} M), (b) $(DH)_2(PhCH_2)Co(py)$ (4.4×10^{-3} M), (c) AdoCbl (4.5×10^{-3} M), (d) and (e) CH_3Cbl (4.5×10^{-3} M) at 298 K after photoirradiation of monochromatized light; (a) and (b): 10 min photoirradiation with $\lambda_{ex} = 370$ nm, the solution contains 10% CH_3CN used as a cosolvent, (c) and (d): 10 min photoirradiation with $\lambda_{ex} = 340$ nm, and (e): 1 min photoirradiation with $\lambda_{ex} = 340$ nm (lane 1) and 520 nm (lane 2).

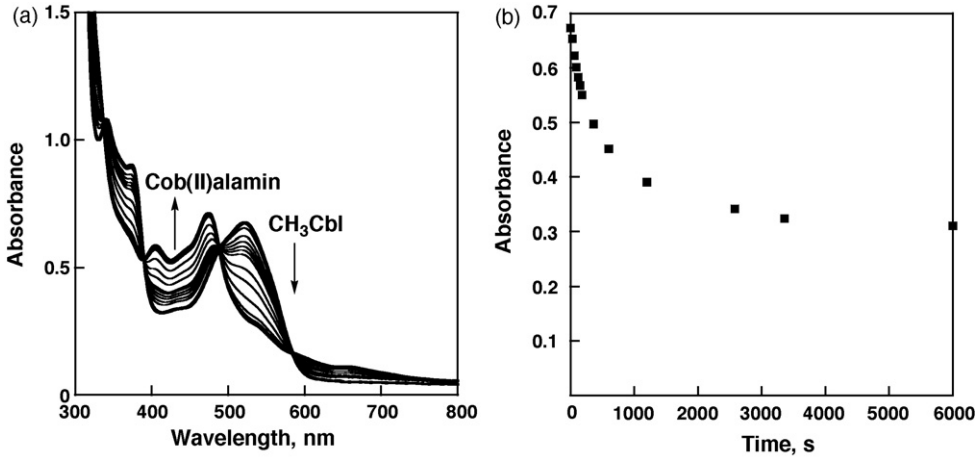


Fig. 3. (a) Absorption spectral change observed in the photochemical reaction of CH_3Cbl (8.4×10^{-5} M) in the presence of GMP (4.5×10^{-1} M) in a deaerated aqueous buffer solution (pH 7.0) under photoirradiation of monochromatized light of $\lambda_{max} = 340$ nm at 298 K. The arrows indicate the direction of change. (b) Plot of the absorbance at 520 nm vs. time.

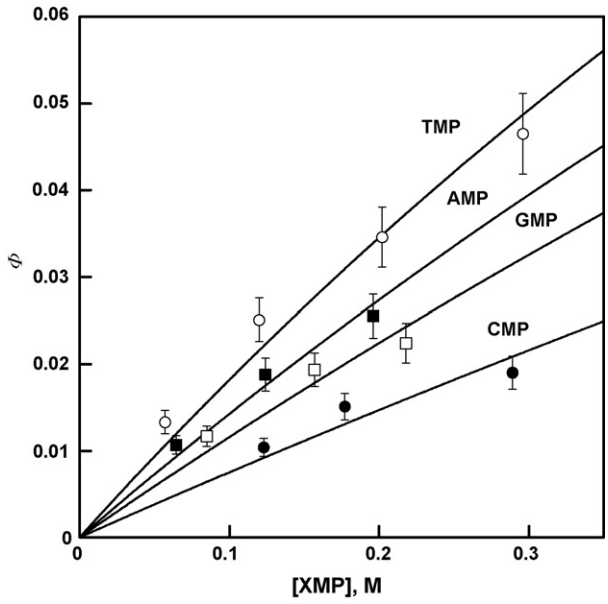
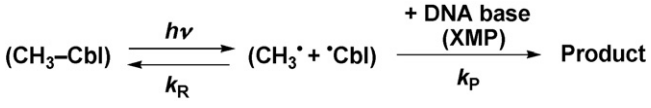


Fig. 4. Plots of Φ vs. concentrations of nucleotides ([XMP]) for the photochemical reactions of CH_3Cbl with GMP, AMP, CMP, and TMP in a deaerated aqueous buffer solution (pH 7.0) at 298 K. The curves represent the best fit to Eq. (1) by using $\alpha = 0.34$ [30,31].

where In is the light intensity

$$\frac{d[CH_3^\bullet + {}^\bullet Cbl]}{dt} = \alpha In - k_P[XMP][CH_3^\bullet + {}^\bullet Cbl] - k_R[CH_3^\bullet + {}^\bullet Cbl] \quad (2)$$

$$\frac{d[Product]}{dt} = k_P[XMP][CH_3^\bullet + {}^\bullet Cbl] \quad (3)$$



Scheme 1.

absorbed by CH_3Cbl. By applying the steady-state approximation (Eq. (4)), the reaction rate is given by Eq. (5), from which Eq. (1) is derived. The curves in Fig. 4 represent the best fit to Eq. (1) by using $\alpha = 0.34$ [30,31].

$$\alpha In - k_P[XMP][CH_3^\bullet + {}^\bullet Cbl] - k_R[CH_3^\bullet + {}^\bullet Cbl] = 0 \quad (4)$$

$$\frac{d[Product]}{dt} = \frac{\alpha In k_P[XMP]}{k_R + k_P[XMP]} \quad (5)$$

On the other hand, the Φ value of the photoreaction of $(DH)_2(CH_3)Co(py)$ ($\lambda_{max} = 370$ nm) with TMP (0.3 M) was determined as 0.06, which was slightly larger than that of CH_3Cbl [32]. In the case of photoreaction of $(DH)_2(PhCH_2)Co(py)$ ($\lambda_{max} = 370$ nm) with TMP, no spectral change of CH_3Cbl was observed. In the case of photoreaction ($\lambda_{max} = 340$ nm) of AdoCbl, no acceleration of the decay rate of the absorbance due to AdoCbl was observed by the presence of DNA nucleotides.

Irradiation of the absorption band of CH_3Cbl in a deaerated aqueous buffer solution (pH 7.0) containing DNA ([DNA nucleotides of calf thymus DNA] = 1.2×10^{-2} M) also resulted in a decrease in the absorbance of CH_3Cbl as shown in Fig. 5. From the plots in Fig. 5b, the quantum yield of the photoreaction of CH_3Cbl with calf thymus DNA (1.2×10^{-2} M) was determined as 0.03.

After completion of the reaction, the gaseous product was analyzed by gas chromatography (GC). Methane, which was formed via homolytic cleavage of the C–C bond in CH_3Cbl, was detected as a product (22–37% yield) (see Table 1).

These results show that the methyl radical generated by the homolytic cleavage of the Co–C bond abstracts hydrogen from all nucleotides. Methyl radical is reported to abstract hydrogen from the sugar moiety of DNA, leading to DNA strand breaks [9,10,19,33]. Using DFT calculations, it was shown that hydrogen abstraction from C1′ position of 2′-deoxyribose moiety was thermodynamically the most feasible [33]. The difference in the reactivity of nucleotides toward methyl radical (Fig. 4) suggests that the hydrogen abstraction rate from C1′ of the sugar moiety is affected slightly by the nucleotide moiety [34]. Since

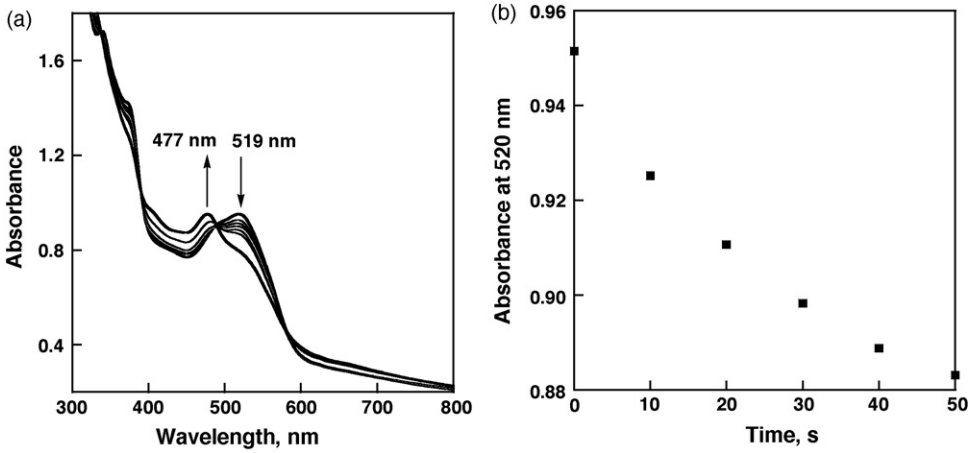


Fig. 5. (a) Absorption spectral change observed in the photochemical reaction of CH_3Cbl (8.0×10^{-5} M) in the presence of calf thymus DNA (1.2×10^{-2} M) in a deaerated aqueous buffer solution (pH 7.0) under photoirradiation of monochromatized light of $\lambda_{max} = 340$ nm at 298 K. The arrows indicate the direction of change. (b) Plot of the absorbance at 520 nm vs. time.

Table 1
Yields of methane formed via reaction of $CH_3^\bullet$ generated from photoexcitation of CH_3Cbl (8.0×10^{-5} M) with DNA nucleotide (4.0×10^{-1} M) or calf thymus DNA (1.2×10^{-2} M)

DNA nucleotides or DNA	Yield of CH_4 (%)[a]
TMP	22
AMP	30
GMP	28
CMP	24
Calf thymus DNA	37

[a] No formation of methane in the absence of nucleotide or calf thymus DNA in these experimental conditions.

methyl radical is known to methylate DNA bases, methyl radical generated by the photoexcitation of CH_3Cbl may also methylate DNA base moiety [35,36]. However, the reactivity in the methylation of nucleosides by methyl radical is reported to be in the order: cytidine > adenosine > guanosine > thymidine [36], which is quite different from the observed reactivity difference in DNA nucleotides in Fig. 4. Thus, the hydrogen abstraction may be the major pathway for the photoreaction of CH_3Cbl with DNA bases in an aqueous solution at pH 7. However, the detailed mechanism and product analysis of the DNA cleavage have yet to be clarified.

The formation of Cob(II)alamin in the photoreaction of CH_3Cbl with TMP was confirmed by ESR spectroscopy. A deaerated aqueous buffer solution (5 mM Tris–HCl (pH 7.0)) containing CH_3Cbl (1.6×10^{-3} M) and TMP (2.3×10^{-1} M) was introduced in an ESR sample tube, which was irradiated with a 1000-W high-pressure mercury lamp through an aqueous filter at 298 K. The ESR spectrum measured at 77 K is shown in Fig. 6, where a typical ESR spectrum of Co(II) species is observed with an anisotropic signal at $g_{||} = 2.00$ and $g_\perp = 2.32$ (Fig. 6). The ESR spectra also reveal the characteristic patterns of eight hyperfine lines due to the interaction of the unpaired electron with one cobalt nucleus ($I = 7/2$) [37,38]. The uniform triplet in each line is attributed to superhyperfine splitting from a nitrogen nucleus ($I = 1$) of the bound benzimidazole. The hyperfine coupling constants (*hfc*) were determined as $A_{||(Co)} = 108$ G and $A_{||}(N) = 18$ G. This value shows good agreement with the reported Co(II) species, Cob(II)alamin [37,38].

The ESR spectrum observed under photoirradiation of an O_2-saturated solution containing $(DH)_2(CH_3)Co(py)$ under

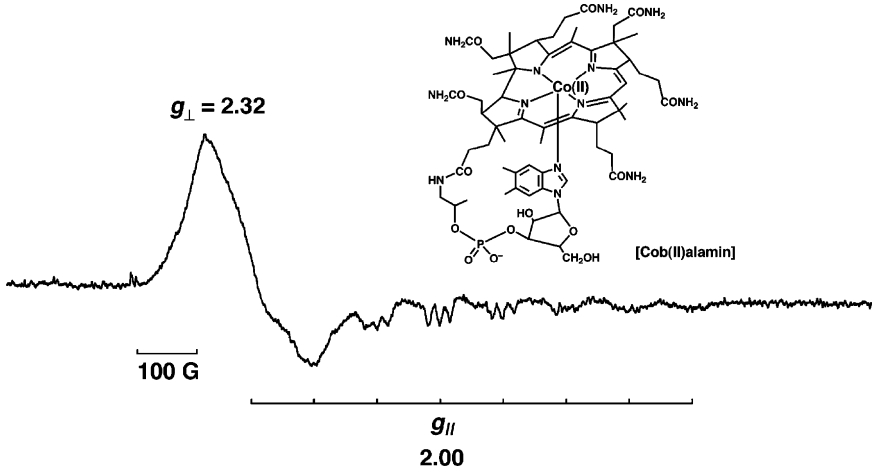


Fig. 6. ESR spectrum observed under photoirradiation of a deaerated aqueous buffer solution (pH 7.0) containing CH_3Cbl (1.6×10^{-3} M) and TMP (2.3×10^{-1} M) with a high-pressure mercury lamp at 298 K, then immediately frozen and measured at 77 K.

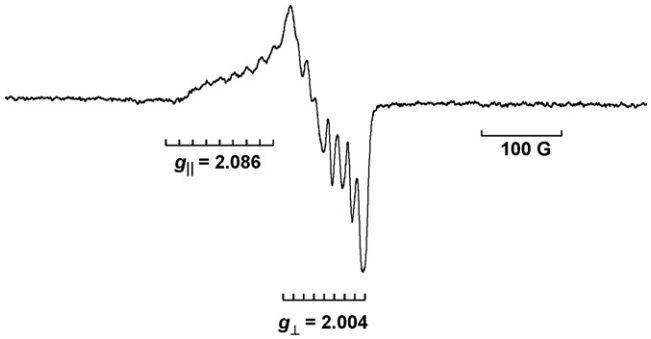


Fig. 7. ESR spectrum observed under photoirradiation of an O_2-saturated propionitrile solution containing $(DH)_2(CH_3)Co(py)$ (2.9×10^{-2} M) with a high-pressure mercury lamp at 298 K, then immediately frozen and measured at 77 K.

photoirradiation with a high-pressure mercury lamp was measured to detect the cobalt-superoxo species (see Fig. 7). The *hfc* values in Fig. 7 are determined as $A_{||}(Co) = 16.6$ G and $A_{\perp}(Co) = 11.6$ G. These values show good agreements with the reported values of a cobalt-superoxo complex, Co(porphyrin)(py)(O_2) [39].

From these results, methyl radical generated by the homolytic cleavage of the cobalt–carbon bond in CH_3Cbl and $(DH)_2(CH_3)Co(py)$ effectively cleaves DNA under anaerobic conditions. On the other hand, under aerobic conditions, methyl radicals are rapidly trapped by dioxygen to form methylperoxyl radical ($CH_3OO^{\bullet}$), which is much less reactive toward DNA [40,41].

When $(DH)_2(CH_3)Co(py)$ is replaced by $(DH)_2(PhCH_2)Co(py)$, the photocleavage of the Co–C bond affords benzyl radical ($PhCH_2^{\bullet}$), which has much lower reactivity than methyl radical [42], thereby leading to no DNA cleavage. On the other hand, the photoexcitation of AdoCbl results in formation of adenosyl radical ($Ado^{\bullet}$), which is known to undergo rapid cyclization to form 5′,8-anhydroadenosine, or abstract a hydrogen to give 5′-deoxyadenosine [17,43]. Such cyclization of $Ado^{\bullet}$ may suppress the DNA cleavage in the photoexcitation of AdoCbl with DNA (vide supra).

4. Conclusions

In conclusion, methyl radical generated from photocleavage of Co–C bond of CH_3Cbl and $(DH)_2(CH_3)Co(py)$ induces effective DNA strand scission via hydrogen abstraction from nucleotides in DNA under anaerobic conditions. Methyl radical produced from CH_3Cbl and $(DH)_2(CH_3)Co(py)$ shows the highest reactivity in the DNA cleavage among B_{12} coenzymes and analogues. Such DNA cleavage by alkyl radicals is strongly suppressed by molecular oxygen, because alky radicals are converted to the much less reactive alkylperoxyl radicals.

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