Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 1063–1067

BIOORGANIC & MEDICINAL CHEMISTRY

Formation of a Fairly Stable Diazoate Intermediate of 5-Methyl-2′-deoxycytidine by HNO_2 and NO, and Its Implication to a Novel Mutation Mechanism in CpG Site

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Received 25 June 2001; accepted 11 October 2001

Abstract—The intermediate produced from 5-methyl-2′-deoxycytidine (^{5me}dCyd) by HNO_2 and NO treatments was isolated and characterized. When 10 mM ^{5me}dCyd was incubated with 100 mM $NaNO_2$ at pH 3.7 and 37 °C, a previously unidentified product was formed. The product was identified as a diazoate derivative of ^{5me}dCyd, 1-(β-D-2′-deoxyribofuranosyl)-5-methyl-2-oxopyrimidine-4-diazoate (^{5me}dCyd-diazoate), on the bases of several measurements including LC/MS. The time course of the concentration change of the diazoate showed a characteristic profile of a reaction intermediate, and the steady state concentration was 2.3 μM (0.023% yield). When an aqueous solution of 10 mM ^{5me}dCyd (10 mL) was bubbled by NO at 37 °C under aerobic conditions holding the pH around 7.4, the diazoate was also generated. The yield of the diazoate was 0.041 μmol (0.041% yield) at 20 mmol of NO absorption. At physiological pH and temperature (pH 7.4, 37 °C), the diazoate was converted to dThd exclusively with a first order rate constant $k = 9.1 \times 10^{-6}\ s^{-1}$ ($t_{1/2} = 21$ h). These results show that the diazoate is generated as a relatively stable intermediate in the reactions of ^{5me}dCyd with HNO_2 and NO and further suggest that the diazoate can be formed in cellular DNA with biologically relevant doses of HNO_2 and NO.

Introduction

In the DNA of many organisms including all studied higher eukaryotes, 5-methyl-2′-deoxycytidine (^{5me}dCyd) exists as a minor component.[1] The ^{5me}dCyd residues in DNA of higher eukaryotes are usually bordered on the 3′-side by a dGuo. In particular, all the CpG dinucleotides of the p53 gene which encodes a multi-functional transcription factor and in which ca. 50% of human cancer contains mutations are known to be methylated in human tissues examined thus far.[2,3] Almost one-fourth mutation of all p53 human tumor mutations and of human genetic diseases is G·C to A·T substitutions at methylated CpG dinucleotides.[2,4] The occurrence of the mutational hot-spots at CpG sites is generally thought to be due to spontaneous hydrolytic deamination of ^{5me}dCyd.[5] Another explanation for this type of mutational hot-spots could be that endogenous compounds induce deamination of ^{5me}dCyd. The candidates responsible for the deamination are nitrous acid (HNO_2) and nitric oxide (NO).[6,7] It has been reported that under aerobic conditions NO deaminates ^{5me}dCyd giving rise to dThd in vitro at a rate of ca. 3-fold greater than that for dCyd.[8]

Recently, we have isolated and characterized an intermediate produced from dCyd by HNO_2 and NO treatments.[9] By spectrometric measurements, the product was identified as a diazoate (–N=N–O$^-$) derivative of dCyd (dCyd-diazoate). The time course of the concentration change of dCyd-diazoate showed a characteristic profile of a reaction intermediate, and dCyd-diazoate concentration was greater than that of dUrd at the initial stage of the reaction. dCyd-diazoate was also generated from dCyd in neutral solution by the NO treatment under aerobic conditions. At physiological pH and temperature (pH 7.4, 37 °C), dCyd-diazoate was

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0968-0896/02/$ - see front matter
PII: S0968-0896(01)00365-0

fairly stable ($t_{1/2}=330$ h). Uracil-DNA glycosylase did not excise the Cyt-diazoate residue from an oligodeoxynucleotide containing this damage. These results show that dCyd-diazoate is generated as a stable intermediate in the reactions of dCyd with HNO_2 and NO, and that the major product is dCyd-diazoate but not dUrd in the initial stage of the reaction. Thus, once formed in vivo, dCyd-diazoate persists for long time in DNA and may act as a major cytotoxic and/or genotoxic lesion.

Since the reaction pathway of nitrosative deamination of ^{5me}dCyd resulting in dThd should be similar to that of dCyd resulting in dUrd, it is expected that a stable diazoate intermediate is also generated in the reaction of ^{5me}dCyd with HNO_2 and NO. However, there is no information available on the intermediates formed by nitrosation of ^{5me}dCyd. We report herein isolation and characterization of the diazoate intermediate of ^{5me}dCyd produced by HNO_2 and NO treatments.

Results

Detection and identification of the intermediate

When 10 mM ^{5me}dCyd was incubated with 100 mM $NaNO_2$ in 1 M sodium acetate buffer (pH 3.7) for 25 s at 37 °C, four peaks appeared in the RP-HPLC chromatogram (Fig. 1). The first peak (retention time, $t_R=5.2$ min) and the second peak ($t_R=11.4$ min) were due to nitrite and unreacted ^{5me}dCyd, respectively. The fourth peak ($t_R=13.1$ min) was attributed to dThd, a deamination product of ^{5me}dCyd, since the retention time and the UV spectrum detected on line for the peak were consistent with those for authentic dThd. An unknown product (termed **1**) was eluted as the third peak ($t_R=12.2$ min), which showed a UV spectrum with $\lambda_{max}=324$ nm (inset of Fig. 1). The reaction kinetics of the formation of **1** and the spontaneous conversion of **1** to dThd showed that **1** was an intermediate of the reaction (vide infra). To identify the intermediate, **1** was isolated by preparative RP-HPLC and several measurements were performed. When **1** was subjected to ion exchange columns, an anion exchange resin (Dowex 1-X8, OH^- form) trapped **1** completely but a cation exchange resin (Dowex 50W-X2, H^+ form) did not. Among all of the expected intermediates in the reaction of ^{5me}dCyd with HNO_2, only the diazoate derivative is an anion (Scheme 1).[9,10] Negative ion APCI-LC/MS using 100% CH_3CN as an eluent showed a signal with m/z 241 for **1**. However, the signal of m/z 269 expected for the intact diazoate anion was not observed in the LC/MS measurement probably due to its instability. In general, aromatic diazoate compounds (R–N=N–O^-) are known to give rise to a strong signal of a [$M^- -28$] fragment which is produced by the loss of a molecular nitrogen through four-centre skeletal rearrangement.[11] Thus, the signal with m/z 241 was attributed to the $-N_2$ product. The on-line UV spectrum of isolated **1** ($\lambda_{max}=324$ nm) was similar to those of the diazoate derivatives of dCyd ($\lambda_{max}=285$ and 310 nm), Guo ($\lambda_{max}=315$ nm), and 9-alkyl Ade ($\lambda_{max}=$ ca. 310 nm).[9,12,13] Combining these data, we have tentatively assigned **1** as a diazoate derivative of ^{5me}dCyd, 1-(β-D-2′-deoxyribofuranosyl)-5-methyl-2-oxopyrimidine-4-diazoate (^{5me}dCyd-diazoate). The structure of ^{5me}dCyd-diazoate is shown in Figure 2.

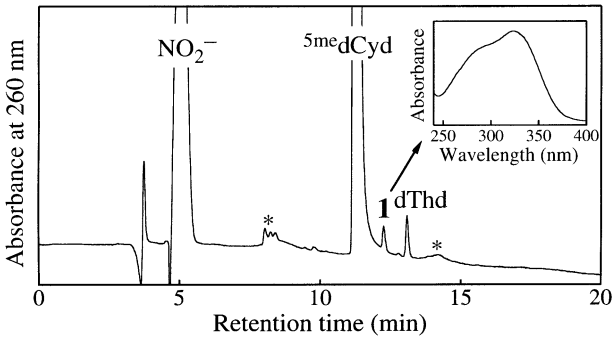
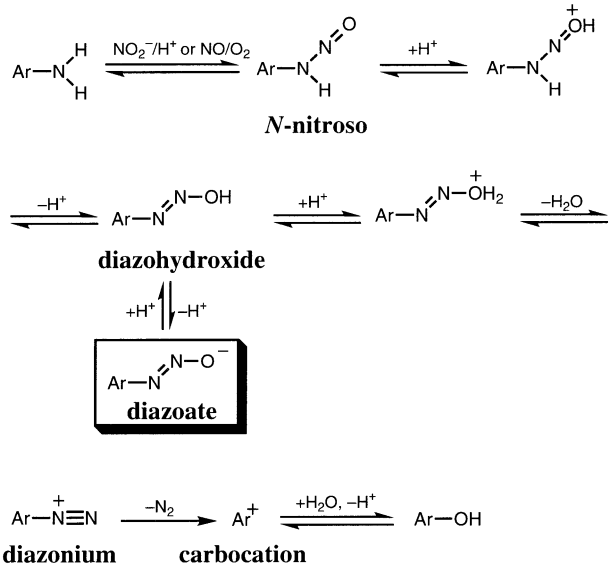


Figure 1. RP-HPLC chromatogram of HNO_2-treated ^{5me}dCyd solution. A solution of 10 mM ^{5me}dCyd was incubated with 100 mM $NaNO_2$ in 1 M sodium acetate buffer at pH 3.7 and 37 °C for 25 s. The reaction was terminated by neutralization with NaOH. The sample was separated by an ODS column (6×250 mm). The eluent was 100 mM triethylammonium acetate buffer (pH 7.0) containing CH_3CN and the flow rate was 1.3 mL/min. The CH_3CN concentration was increased from 0 to 20% for 20 min in a linear gradient mode. Inset is an on-line detected UV spectrum of isolated **1**. The peaks indicated by asterisks are impurities present in starting ^{5me}dCyd.

Reaction kinetics for HNO_2 treatment

The time courses of the concentration changes in ^{5me}dCyd-diazoate and dThd are shown in Figure 3 when 10 mM ^{5me}dCyd was incubated with 100 mM $NaNO_2$ in 1 M sodium acetate buffer (pH 3.7) at 37 °C. The concentration of ^{5me}dCyd-diazoate increased rapidly in the early stage of the reaction and reached a plateau, showing a characteristic profile of a reaction intermediate. At the incubation time of 5 min, the concentrations of ^{5me}dCyd-diazoate and dThd were 2.3 and 36.7 μM, respectively. The concentration of ^{5me}dCyd-deazoate was slightly greater than that of dThd up to 15 s.



Scheme 1. Proposed reaction pathways for the nitrosative deamination of aromatic amines by HNO_2 and NO under aerobic conditions.

Formation of the diazoate by NO

In an open vessel, 10 mM ^{5me}dCyd was dissolved in 10 mL of 100 mM sodium phosphate buffer (pH 7.4) at 37 °C, and NO (99.8%), which was run through a soda lime, was bubbled at the flow rate of 1 mL/s through a glass frit into the well stirring solution under aerobic conditions. The pH of the solution was maintained at 7.4±0.4 by the titration of 1 M NaOH throughout the reaction. The RP-HPLC analysis of the reaction mixture revealed that ^{5me}dCyd-diazoate was generated in addition to dThd. Figure 4 shows the plots of the amounts of ^{5me}dCyd-diazoate and dThd against the mole of the absorbed NO (measured as the mole of NaOH required to neutralize the solution). Similar to the HNO_2 treatment (Fig. 3), the concentration change in ^{5me}dCyd-diazoate showed a characteristic profile of a reaction intermediate. The yields of ^{5me}dCyd-diazoate and dThd were 0.041 and 1.021 μmol, respectively, at 20 mmol absorption of NO. It took 30 min to absorb 20 mmol of NO under the present reaction conditions.

Stability of the diazoate intermediate

To clarify the stability of the diazoate, the isolated ^{5me}dCyd-diazoate was incubated under physiological conditions and the reaction was monitored by RP-HPLC. In 100 mM sodium phosphate buffer (pH 7.4) at 37 °C, ^{5me}dCyd-diazoate disappeared giving rise to dThd with the incubation time. The plot of the concentration of ^{5me}dCyd-diazoate against the incubation time followed the first order kinetics (Fig. 5). The rate constant of the reaction was evaluated as $(9.1\pm0.3)\times10^{-6}$ s^{-1} ($t_{1/2}$ = ca. 21 h) from the slope of the four independent experiments. There was no indication of products other than dThd in the RP-HPLC chromatogram for the hydrolysis of ^{5me}dCyd-diazoate.

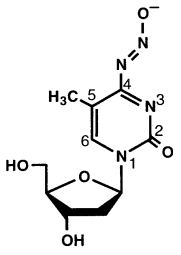


Figure 2. The structure of **1**, a diazoate derivative of ^{5me}dCyd (^{5me}dCyd-diazoate).

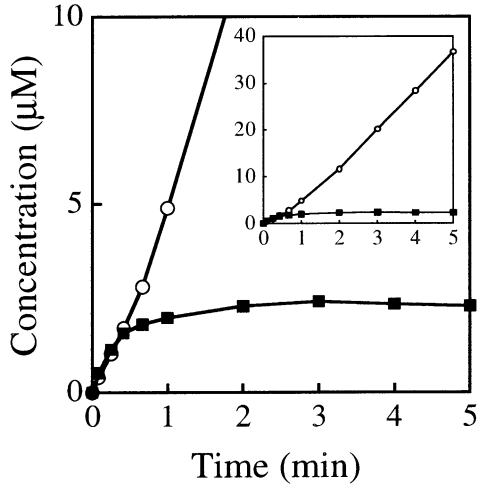


Figure 3. The time courses of the concentration changes in ^{5me}dCyd-diazoate (closed square) and dThd (open circle) when ^{5me}dCyd (10 mM) was incubated with 100 mM $NaNO_2$ in 1 M sodium acetate buffer at pH 3.7 and 37 °C. The concentration was determined by RP-HPLC analysis. The inset shows the same data with an expanded ordinate.

Discussion

In the present study, it was shown that a fairly stable diazoate derivative was produced as an intermediate in the reaction of ^{5me}dCyd with nitrite under mild acidic conditions. The concentration of the diazoate increased rapidly in the early stage of the reaction and was greater than that of dThd up to the reaction time of 15 s (Fig. 3). The steady state concentration (at an incubation time of 5 min) of ^{5me}dCyd-diazoate was 2.3 μM (0.023% yield). This value was 1/15 of that of dCyd-diazoate (at an incubation time of 60 min) when dCyd was incubated with nitrite under the same reaction conditions.[9] However, the total concentration of ^{5me}dCyd-diazoate and dThd (39.0 μM, 0.390% yield) at the reaction time of 5 min, was comparable to that of dCyd-diazoate and dUrd (32.0 μM, 0.320% yield) generated from dCyd at the same reaction time. These results show that ^{5me}dCyd reacts with HNO_2 at a rate similar to that of dCyd and ^{5me}dCyd-diazoate is the dominant species at the initial stage of the reaction. Similarly, ^{5me}dCyd-diazoate was produced from ^{5me}dCyd in neutral solution by NO

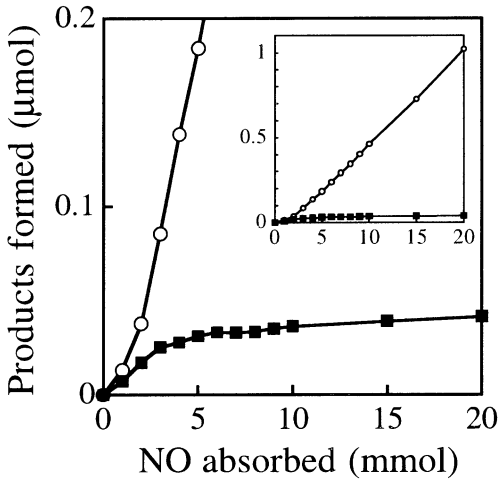


Figure 4. Correlation of the amounts of ^{5me}dCyd-diazoate (closed square) and dThd (open circle) with that of absorbed NO. In an open vessel, 10 mM ^{5me}dCyd was dissolved in 10 mL of 100 mM sodium phosphate buffer (pH 7.4) at 37 °C, and NO was bubbled at the flow rate of 1 mL/s through a glass frit into the well stirring solution under aerobic conditions. The pH was held in the range of 7.4±0.4 by the titration of 1 M NaOH. The amount of absorbed NO was calculated from that of added NaOH. The concentrations of ^{5me}dCyd-diazoate and dThd were determined by RP-HPLC analysis. The inset shows the same data with an expanded ordinate.

bubbling under aerobic conditions. The yield of ^{5me}dCyd-diazoate was 0.041 μmol (0.041% yield) at 20 mmol of NO absorption which was ca. 1/4 of that of dCyd-diazoate.[9] However, the total yield of ^{5me}dCyd-diazoate and dThd was 1.062 μmol (1.062% yield) which was 3.2-fold greater than that of dCyd-diazoate and dUrd generated from dCyd under the same conditions. The results indicate that, in the successive reactions [^{5me}dCyd (dCyd) → ^{5me}dCyd-diazoate (dCyd-diazoate) → dThd (dUrd)], the reaction rate of the first reaction is 3.2-fold greater for ^{5me}dCyd than dCyd. In the present study, 96% of the generated ^{5me}dCyd-diazoate was converted to the final product (dThd) during the 30 min reaction (20 mmol absorption of NO), indicating that the conversion of ^{5me}dCyd-diazoate to dThd was fairly rapid. On the other hand, another experiment of this study demonstrated that the half-life of the isolated ^{5me}dCyd-diazoate was 21 h in 100 mM sodium phosphate buffer (pH 7.4) at 37 °C (Fig. 5). If the conversion of ^{5me}dCyd-diazoate to dThd had occurred only by attack of a water molecule alone in the NO bubbled solution, a much higher concentration of ^{5me}dCyd-diazoate should have been accumulated throughout the reaction. Thus, it is very likely that the conversion of ^{5me}dCyd-diazoate to dThd during the NO treatment was accelerated by the attack of coexisting species such as NO or other nitrogen oxides generated from NO. In the present study, the concentration of ^{5me}dCyd-diazoate was lower than that of dThd even at the initial stage of the reaction (i.e., 1 mmol NO absorption), although the concentration of ^{5me}dCyd-diazoate approached that of dThd with the decrease in the amount of NO employed (Fig. 4). As mentioned above, this may also be due to the accelerated conversion of ^{5me}dCyd-diazoate to dThd by the coexisting nitrogen species. It is reported that cellular NO concentrations are a several hundred nM even after electrical or chemical stimulation.[14,15] Therefore, the cellular NO concentration is at least five orders lower than those employed in the present in vitro study. Considering the actual cellular NO concentration, it is very unlikely that ^{5me}dCyd-diazoate further reacts with NO or other nitrogen oxides generated from NO, yielding dThd in cells. Accordingly, the major product will be ^{5me}dCyd-diazoate but not dThd at a physiological exposure level of NO.

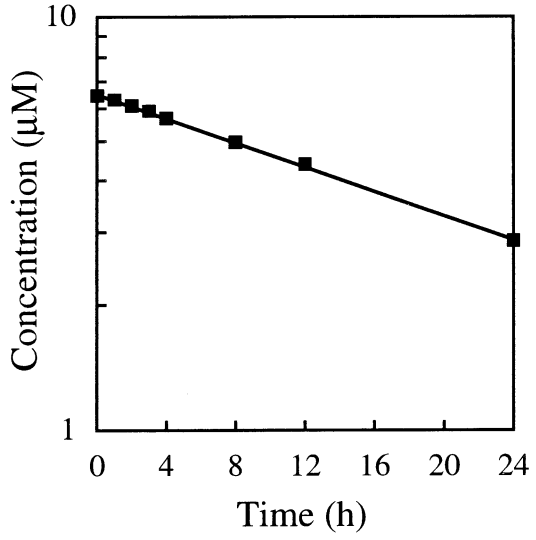


Figure 5. An exponential plot of the concentration change in ^{5me}dCyd-diazoate as a function of the incubation time. The isolated ^{5me}dCyd-diazoate was incubated in 100 mM sodium phosphate buffer at pH 7.4 and 37 °C. The concentration of ^{5me}dCyd-diazoate was determined by RP-HPLC analysis.

^{5me}dCyd-diazoate was converted to dThd by spontaneous hydrolysis under physiological conditions. In general, diazoate compounds (Ar–N=N–O$^-$) would be spontaneously converted to several intermediates such as diazoniums (Ar–N≡N$^+$) and carbocations (Ar$^+$) in neutral solutions as shown in Scheme 1.[9,10] The conversion of the diazoate of ^{5me}dCyd to dThd should be initiated by protonation and terminated by a nucleophilic reaction of a water molecule to C4 cation or C4 diazonium species of ^{5me}dCyd. At physiological pH and temperature (pH 7.4 and 37 °C), ^{5me}dCyd-diazoate had a half-life of 21 h which was ca. 1/16 of that ($t_{1/2}$ = 330 h) of dCyd-diazoate. In double-stranded DNA, bases are protected from protonation and an attack of H_2O relative to those in single-stranded DNA or nucleosides. For instance, the rate of hydrolytic deamination of dCyd in double-stranded DNA was ca. 140-fold smaller than that in single-stranded DNA.[16] Analogously, ^{5me}dCyd-diazoate generated in double-stranded DNA would be protected from protonation and an attack of H_2O, and may remain intact for a considerably long period.

Methylated CpG sites in DNA generate a high frequency of mutations and constitute mutational hotspots both in human cancer and in human genetic diseases.[2,4] Deamination of ^{5me}dCyd at the methylated CpG sites is considered to be the major cause of these mutations.[5] Deamination of ^{5me}dCyd results in dThd and generates a G·T mismatch which, if not repaired, leads to a G·C to A·T transition. However, thymine–DNA glycosylase exists in human cells and specifically excises thymine from G·T mispairs in CpG sites in DNA.[17,18] Thus, the conventional mutation mechanism related to deamination of ^{5me}dCyd is exclusively attributable to the insufficient repair by thymine–DNA glycosylase of dThd generated from ^{5me}dCyd by both hydrolytic and nitrosative deamination. The present study has shown that a significant amount of the fairly stable diazoate intermediate, ^{5me}dCyd-diazoate, exists in the nitrosative reaction of ^{5me}dCyd by HNO_2 and NO before conversion to dThd. If ^{5me}dCyd-diazoate is formed in DNA and is not excised by thymine–DNA glycosylase or other repair enzymes, the lesion remains for a certain period. The diazoate escaped from repair may cause a mutation directly when DNA replication passes through the lesion. Alternatively, if the diazoate is hydrolyzed and results in dThd during DNA replication, repair of this lesion becomes more difficult. Especially, when the conversion to dThd occurs after duplex DNA is separated to single strands, dThd can not be excised by any repair systems since dThd is a normal component of DNA. This is in contrast to the fact that dUrd resulting from deamination of dCyd is excised by uracil–DNA glycosylase even in single-stranded DNA.[19,20]

Conclusion

We have demonstrated that a diazoate intermediate is formed in the reaction of ^{5me}dCyd with HNO_2 and NO. The diazoate was relatively stable at physiological pH and temperature. These results bring forward another possible scheme of mutation caused by nitrosative deamination of ^{5me}dCyd in which ^{5me}dCyd-diazoate plays a unique role. To evaluate biological importance of ^{5me}dCyd-diazoate, further studies are required on in vivo formation of the diazoate.

Experimental

Materials

dThd and 5-methyl-2′-deoxycytidine (^{5me}dCyd) were purchased from Sigma (St. Louis, MO, USA). All other chemicals of reagent grade were purchased from Wako Pure Chemicals (Osaka, Japan) or Nacalai Tesque (Osaka, Japan), and used without further purification. Water was purified with a Millipore Milli-QII deionizer.

RP-HPLC conditions

The HPLC system consisted of Tosoh DP-8020 pumps and a PX-8020 system controller. On-line UV spectra were obtained with a Tosoh PD-8020 UV–vis photodiode-array detector. For reversed phase (RP-) HPLC, a Cosmosil 5C18-AR-II octadecylsilane (ODS) column (Nacalai Tesque, 6×250 mm, particle size 5 μm) was used. The eluent was 100 mM triethylammonium acetate buffer (pH 7.0) containing CH_3CN. The CH_3CN concentration was increased from 0 to 20% for 20 min in a linear gradient mode. HPLC analysis was performed at room temperature with a flow rate of 1.3 mL/min.

Spectrometric measurements

Negative-ion atmospheric pressure chemical ionization (APCI-) LC mass spectra were obtained by a Hitachi M-2000 MS system. The sample was directly injected into the MS system by an HPLC pump without separation columns. The following LC/MS conditions were used: eluent, 100% CH_3CN (isocratic); flow rate, 0.5 mL/min; vaporization temperature, 300 °C; desolvation temperature, 305 °C; drift voltage, −195 V.

Quantitative procedures

The concentrations of products were evaluated from integrated peak areas of the HPLC chromatogram and the molar extinction coefficients at 260 nm. The ε_{260} value of ^{5me}dCyd-diazoate was estimated as $7.4\times10^3\,M^{-1}\,cm^{-1}$, assuming that ^{5me}dCyd was converted to dThd exclusively by the incubation in 100 mM sodium phosphate buffer (pH 7.4) at 37 °C. The ε_{260} values of ^{5me}dCyd and dThd were 5.9×10^3 and $8.9\times10^3\,M^{-1}\,cm^{-1}$, respectively.[21] In the quantitative analysis of the reaction products, the initial RP-HPLC peak area was used as a standard.

Acknowledgements

This work was partly supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan [to K.M. (11101001, 10151219, 10878092, and 12480173), and H.I.], and by a Grant from 'Research for the Future' Program of Japan Society for the Promotion of Science (JSPS-RFTF97I00301).

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