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Identification of 3,N^4-Etheno-5-methyl-2′-deoxycytidine in Human DNA: A New Modified Nucleoside Which May Perturb Genome Methylation

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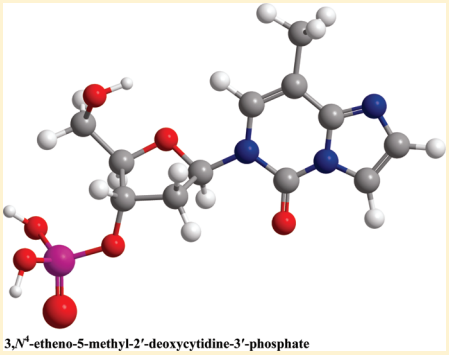
Supporting Information

ABSTRACT: Methylation of cytidine at dCpdG sequences regulates gene expression and is altered in many chronic inflammatory diseases. Inflammation generates lipid peroxidation (LPO) products which can react with deoxycytidine, deoxyadenosine, and deoxyguanosine in DNA to form pro-mutagenic exocyclic etheno-nucleoside residues. Since 5-methyl-2′-deoxycytidine (5mdC) residues exhibit increased nucleophilicity at N3, they should be even better targets for LPO products. We synthesized and characterized 3,N^4-etheno-5-methyl-2′-deoxycytidine-3′-phosphate and showed that LPO products can indeed form the corresponding etheno-5mdC (ε5mdC) lesion in DNA *in vitro*. Our newly developed ^{32}P-postlabeling method was subsequently used to detect ε5mdC lesions in DNA from human white blood cells, lung, and liver at concentrations 4–10 times higher than that observed for etheno adducts on nonmethylated cytidine. Our new detection method can now be used to explore the hypothesis that this DNA lesion perturbs the DNA methylation status.



3,N^4-etheno-5-methyl-2′-deoxycytidine-3′-phosphate

INTRODUCTION

DNA methyl transferases (DNMT) catalyze the covalent attachment of a methyl group to the C5 position of cytosine bases in DNA to form 5-methyl-2′-deoxycytidine (5mdC) residues.[1] Most of these methylations occur at the -p5′dC3′p5′dG3′p- sequence positions and are found to be related to gene expression. Such dCpdG sequences in the promoter region of inactive genes are usually methylated, whereas demethylation at these sites often results in increased transcriptional activity. Both hypomethylation of the whole DNA (global hypomethylation) and site-specific hypermethylation have been observed in common human chronic degenerative diseases, including cancer.[2] Currently, it is still largely unknown how the balance between methylation and demethylation rates is maintained or altered. No correlations have been observed between the degrees of hypo- and hypermethylation, suggesting that the mechanisms responsible for alterations of methylation patterns are independent of each other.[3−5] Thus, the overall pattern of DNA methylation is thought to be the result of a dynamic equilibrium between the rates of DNA methylation and demethylation. Disturbance of this balance may lead to changes in gene expression and cell behavior which initiate or promote the early stages of carcinogenesis.

Although global hypomethylation has been extensively studied, it is still unclear what processes underlie this phenomenon. It is very difficult to discriminate between (a) reduction or inhibition of the methylation of a newly synthesized DNA strand and (b) increased removal of methyl groups from previously methylated dCpdG sequences. Evidence for the existence of an active DNA demethylation process has been obtained,[6,7] but other studies have suggested that hypomethylation may also result when DNMTs are prevented from accessing DNA.[8] Continuous formation of damaged DNA and its repair during chronic inflammation may be involved in the latter process. The structural characteristics of a damaged dCpdG sequence may hinder site-specific access and methylation by a DNMT. It has been shown that oxidative DNA lesions at certain positions in a dCpdG unit can also result in *increased* DNA methylation,[9] probably because some proteins involved in the methylation machinery can be considered to be "ancient" DNA repair enzymes. For instance, the enzymes MBD4 (methyl-dCpdG binding domain protein 4)[10] and thymine DNA glycosylase[11] can both be involved in the repair of mismatches at dCpdG sites. Thymine DNA glycosylase is additionally involved in the repair of the etheno-adduct residues 3,N^4-etheno-2′-deoxycytidine (εdC) in damaged DNA.[11,12]

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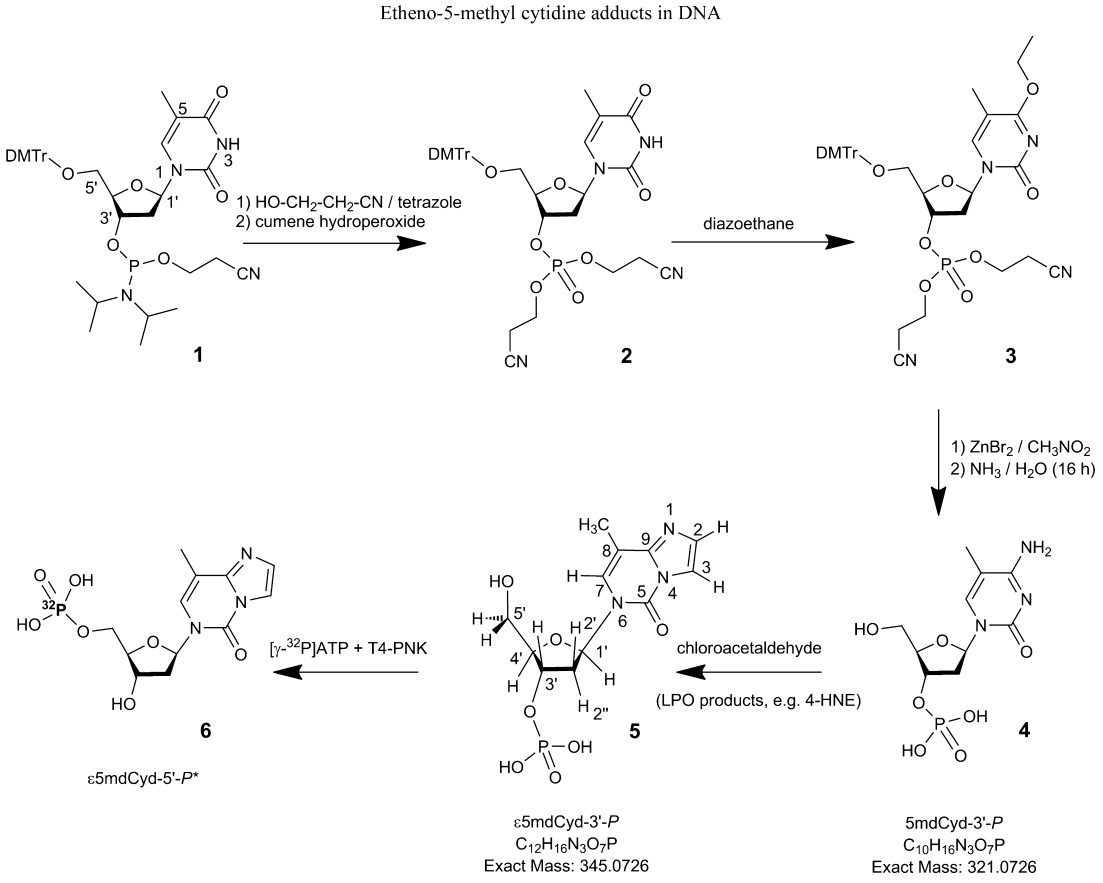
Scheme 1. Synthesis of 3,N^4-Etheno-5-methyl-2′-deoxycytidine-3′-phosphate (5) and the ^{32}P-Labeled 5′-Phosphate (6)[a]



[a]A brief description of the synthesis is provided in Experimental Procedures; details are provided in Supporting Information. The starting compound **1** is available commercially. DMTr = 4,4′-dimethoxytrityl protecting group; T4-PNK = T4 polynucleotide kinase. The conventional atom numbering scheme for ribose and the pyrimidine bases has been used. For etheno adducts **5** and **6**, the atom numbering in the modified base corresponds to the IUPAC systematic name 8-methylimidazo[1,2-*c*]pyrimidin-5(6H)-one for the fused ring system. The adduct formation step **4** → **5** can also be carried out with lipid peroxidation products such as 4-HNE, derived from arachidonic acid, and represents the primary mechanism for adduct formation in DNA *in vivo*. Step **5** → **6** represents the ^{32}P-postlabeling reaction used for ultrasensitive detection of etheno adducts in DNA digests.

Promutagenic etheno-DNA adducts, which include the modified nucleoside residues εdC and 1,N^6-etheno-2′-deoxyadenosine (εdA), are formed *inter alia* by the major lipid peroxidation (LPO) product *trans*-4-hydroxy-2-nonenal (4-HNE).[13] Reaction products of 4-HNE with 5mdC residues have not been investigated up to now, but their existence has been postulated since the nucleophilicity of the N3 position toward electrophilic LPO products is enhanced in 5mdC relative to dC residues.

Here we report for the first time that the exocyclic adduct 3,N^4-etheno-5-methyl-2′-deoxycytidine (ε5mdC) is indeed formed when DNA containing 5mdC residues is incubated with electrophilic LPO-derived products. The new model nucleotide 3,N^4-etheno-5-methyl-2′-deoxycytidine-3′-phosphate (ε5mdCyd-3′-*P*) with modified cytosine base was synthesized and structurally characterized by mass spectrometry (MS) and nuclear magnetic resonance (NMR). An ultrasensitive ^{32}P-postlabeling method was developed which facilitates the detection and quantitation of etheno adducts (εdA, εdC, ε5mdC) as labeled nucleotides in digests of DNA from human and animal tissues.

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies (Mabs). The Mabs used in this study were provided by M. F. Rajewsky (Institute of Cell Biology, University of Essen, Essen, Germany), and their characteristics have been reported previously.[14] The Mab EM-C-1 was raised to recognize the modified nucleoside εdCyd,[14] and it was expected that EM-C-1 will also recognize the structurally similar ε5mdCyd nucleoside and the corresponding phosphate nucleotides. Similarly, Mab EM-A-1 was raised to recognize the nucleoside εdA.

3,N^4-Etheno-5-methyl-2′-deoxycytidine-3′-phosphate (5). The synthesis route is summarized in Scheme 1, and details are presented in the Supporting Information. As previously described,[15] commercially available 5′-*O*-(4,4′-dimethoxytrityl)-thymidine-3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (**1**) (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) was first converted to 5′-*O*-(4,4′-dimethoxytrityl)-thymidine-3′-bis(2-cyanoethyl)phosphate (**2**) and then to O^4-ethyl-5′-*O*-(4,4′-dimethoxytrityl)-thymidine-3′-bis(2-cyanoethyl)phosphate (**3**). The dimethoxytrityl protecting group was removed from the 5′ site using $ZnBr_2$. Normally, the deprotection of the 3′-phosphate is performed by incubation with ammonia for less than 3 h. However, in Scheme 1 the incubation was prolonged to 16 h, and the O^4-ethyl moiety on thymine was efficiently replaced by an NH_2 group in almost 100% yield. After the removal of ammonia from the reaction mixture *in vacuo*, the solution was lyophilyzed, and the residue was subjected to reverse-phase HPLC (instrumentation, Hewlett-Packard, C18-reverse phase column; mobile phase gradient, 0 to 50% acetonitrile in water over 30

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min; flow rate, 1 mL/min) to give pure 5-methyl-2′-deoxycytidine-3′-phosphate (5mdCyd-3′-*P*, **4**). This compound was subsequently incubated with 1 M chloroacetaldehyde to form the etheno-adduct 3,N^4-etheno-5-methyl-2′-deoxycytidine-3′-phosphate (ε5mdCyd-3′-*P*, **5**), which was purified by reverse-phase HPLC using the method given above. For $C_{12}H_{16}N_3O_7P$, the exact mass M = 345.0726; LC-ESI-MS (*m*/*z*): $[M - H]^-$ calcd. 344.0653; found, 344.1; $[2M-H]^-$ calcd. 689.1379; found, 689.1; $[M-(C_7H_7N_3O)-H]^-$ calcd. 195.0064; found, 195.0 (neutral loss of the modified base ε5mCyt).

The ^{1}H NMR data summarized in Table 1 were obtained at 500 MHz (Bruker AM-500 spectrometer, Bruker BioSpin GmbH, Rheinstetten, Germany) and 30 °C with a 0.5 mg sample in 0.4 mL of D_2O. Chemical shifts were referenced to the residual HDO signal, defined as 4.725 ppm. A series of homodecoupling experiments were performed to assign chemical shifts and couplings. A first-order analysis of J_{HH} values was made with the multiplet analyis routines in Bruker's TopSpin software after Lorentz−Gauss resolution enhancement.

Table 1. ^{1}H NMR Data for 3,N^4-Etheno-5-methyl-2′-deoxycytidine-3′-phosphate (5)[a]

pos.[b]	δ_H (ppm)	mult.	J_{HH} (partner) in Hz
1′	6.560	dd	6.85 (2′a); 6.40 (2′b), 0.5 (7)
2′a (2′)	2.504	ddd	−14.27 (2′b); 6.82 (3′)
2′b (2″)	2.686	ddd	3.79 (3′)
3′	4.834	dddd	3.74 (4′); 7.28 (P)
4′	4.267	ddd	4.84 (5′a); 3.29 (5′b)
5′a (5″)	3.852	dd	−12.61 (5′b)
5′b (5′)	3.921	dd	
2	7.592	d	2.00 (3)
3	7.924	d	
7	7.679	q br	1.26 (8-Me)
8-Me	2.316	d	

[a]Five hundred megahertz, 0.5 mg in 0.4 mL of D_2O, 30 °C, shifts relative to HDO = 4.725 ppm; *J* couplings were estimated from first-order analysis with a precision of ca. ± 0.05 Hz. ^{1}H NMR spectrum for 3,N^4-etheno-5-methyl-2′-deoxycytidine-3′-phosphate (**5**) is shown in Supporting Information. [b]Atom numbering as in Scheme 1 and Barrio et al.;[23] for nonequivalent methylene protons, the symbols a and b refer to the lower and higher ppm values; primes (′ and ″) follow the conventions used for NMR of deoxyribose derivatives.[22] Positions (pos.), 7 and 8 in the etheno-cytosine base correspond to pos. 6 and 5 in cytosine, respectively, so that 8-Me in the adduct corresponds to 5-Me in the normal nucleotide.

3,N^4-Etheno-5-methyl-2′-deoxycytidine-5′-[^{32}P]phosphate (6). Compound **5** was phosphorylated and ^{32}P-labeled at the 5′ site using [γ-^{32}P]ATP (>5000 Ci/mmol) and T4 polynucleotide kinase at pH 6.8. Under these conditions, the kinase also behaves as a 3′-phosphatase, thus removing the phosphate group from the 3′ site while labeling the 5′ site.[16] The labeling procedure was performed before and after purification of **5** on immunoaffinity columns prepared with the Mab EM-C-1. The ^{32}P-labeled 5′-phosphates were resolved on polyethyleneimine TLC plates using two-dimensional chromatography [D1 = 1 M acetic acid (pH 3.5) and D2 = saturated ammonium sulfate (pH 3.5)]. After autoradiography, adduct spots were marked and cut out, and the radioactivity was measured in a liquid scintillation counter. Radiolabeling of the standard ε5mdCyd-3′-*P* (**5**) before and after immuno-purification yielded similar results, indicating that the immunoaffinity columns prepared with EM-C-1 indeed recognized and retained ε5mdCyd-3′-*P*.

^{32}P-Labeling of Nucleotides after DNA Hydrolysis. Native or pretreated (see below) DNA samples (25 μg) were hydrolyzed to nucleotide 3′-phosphates using micrococcal endonuclease and spleen phosphodiesterase.[16] Normal nucleotides were quantitated by HPLC using an aliquot of the digest, while etheno adducts were enriched on immunoaffinity columns prepared from the Mabs EM-A-1 (recognizes εdAdo-3′-*P*) and EM-C-1 (recognizes εdCyd-3′-*P* and ε5mdCyd-3′-*P*). Since EM-C-1 was used to bind the etheno adduct of both methylated and nonmethylated cytidine, two consecutive columns with EM-C-1 were used to increase total binding capacity, to minimize the consequences of competitive binding and to ensure the highest possible retention of both εdC and ε5mdC. The etheno adducts and the internal standard (2′-deoxyuridine-3′-phosphate) were converted to radiolabeled 5′-phosphates (5′-*P**) using [γ-^{32}P]ATP and T4-polynucleotide kinase at pH 6.8. The 5′-phosphates were resolved on polyethyleneimine TLC plates as described above. The absolute amounts of the adducts were quantitated by liquid scintillation using standards, and the relative levels or frequencies of the adduct nucleoside residues (ε5mdC, εdC and εdA) in DNA were calculated from the measured amounts of the nonadduct nucleotides and listed in Table 2 as adduct residues per 10^9 parent residues or per 10^9 total residues. The content of 5mdCyd-3′-*P* in the digest was determined by labeling an aliquot of the digest after 1000-fold dilution in water and subsequent separation of 5mdCyd-5′-*P** and dCyd-5′-*P** by TLC using the same conditions as those described above (see Figure 2B).

***In Vitro* Generation of Etheno Adducts in DNA by Chloroacetaldehyde and by LPO Products of Arachidonic Acid.** Chloroacetaldehyde (CAA) is one of the metabolites of the human carcinogen vinyl chloride, and it reacts with DNA bases to

Table 2. Etheno-nucleoside Adduct Residue Levels (Mean ± SD, *n* = 3) in Calf Thymus DNA[a]

		etheno-nucleoside residue levels [per 10^9 parent (P) or total (T) residues]			ratios	
agent [mM]	norm.[b]	εdA	εdC	ε5mdC	ε5mdC/εdA	ε5mdC/εdC
CAA [0]	P	25 ± 16	403 ± 149	1362 ± 490	54.5	3.38
	T	7 ± 5	85 ± 31	13 ± 5	1.86	0.15
CAA [2]	P	234 ± 85	469 ± 278	3541 ± 1541	15.1	7.55
	T	68 ± 25	98 ± 58	35 ± 15	0.51	0.36
CAA [20]	P	776 ± 102	4362 ± 1831	28841 ± 3853	37.2	6.61
	T	225 ± 30	916 ± 384	285 ± 38	1.27	0.31
AA [0]	P	16 ± 8	428 ± 44	1456 ± 227	91.0	3.40
	T	5 ± 2	90 ± 9	14 ± 2	2.80	0.16
AA [0.25]	P	30 ± 21	653 ± 180	2062 ± 1172	68.7	3.16
	T	9 ± 6	137 ± 38	20 ± 12	2.22	0.15
AA [0.50]	P	74 ± 23	1260 ± 789	3768 ± 1054	50.9	2.99
	T	21 ± 7	265 ± 166	37 ± 10	1.76	0.14

[a]DNA incubated *in vitro* with various concentrations of CAA or with LPO products of arachidonic acid (AA), as described in the Experimental Procedure section. [b]Normalization (norm.): adduct residue levels expressed as number per 10^9 nucleosides of the parent type (P) or total nucleosides (T).

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form labile hydroxyethano-derivatives which subsequently dehydrate to give the etheno adducts. Calf thymus DNA (200 μg) in 1 mL of 0.1 M Tris-HCl buffer (pH 7.1) was incubated with increasing concentrations of CAA (0, 2, and 20 mM) for 30 min at 37 °C. DNA was precipitated with two volumes of cold ethanol after the addition of 1/30 volumes of 3 M sodium acetate (pH 5.3). The DNA was then washed with 70% ethanol, dried, and stored at −20 °C as 25-μg aliquots.

LPO products from arachidonic acid were generated in the presence of ferrous sulfate. The reaction mixture contained 0, 0.25, or 0.5 mM arachidonic acid, 0.75 mM $FeSO_4$ (freshly prepared), 0.1 mM NADPH, and 200 μg of calf thymus DNA in 1 mL of 0.1 M Tris-HCl buffer (pH 7.1). Reactions were allowed to proceed at 37 °C for 30 min and were completed by heating for 30 min at 80 °C. The mixtures were dialyzed overnight against doubly distilled water. DNA was precipitated, washed, dried, and stored at −20 °C as 25-μg aliquots, as described above.

Levels of 3,N^4-Etheno-5-methyl-2′-deoxycytidine in DNA from Human Tissues. DNA from (a) two human white blood cell samples with predetermined high εdA and εdC levels, obtained from female volunteers on a very high ω-6 polyunsaturated fatty acid diet,[17] (b) 10 nontumorous lung samples from nonsmoking lung cancer patients,[18] and (c) two asymptomatic human liver samples with known adduct levels of εdA and εdC[16] were analyzed for the presence of ε5mdC using our immuno-enriched ^{32}P-postlabeling method as described above.

RESULTS

Synthetic Reference: 3,N^4-Etheno-5-methyl-2′-deoxycytidine-3′-phosphate. The title reference compound ε5mdCyd-3′-*P* (**5**) was synthesized according to Scheme 1, starting from the commercially available thymidine phosphoramidite **1**, as described briefly in the Experimental Procedures section and in more detail in Supporting Information. Note: the atom numbering scheme for the modified base in compound **5** corresponds to IUPAC nomenclature[19] where, for example, 3,N^4-etheno-5-methyl-2′-deoxycytidine (ε5mdCyd) is defined as 8-methyl-6-(2′-deoxy-β-D-ribofuranosyl)-imidazo[1,2-*c*]-pyrimidin-5(6*H*)-one or, equivalently, 5,6-dihydro-8-methyl-5-oxo-6-(2′-deoxy-β-D-ribofuranosyl)-imidazo[1,2-*c*]pyrimidine.

LC-MS chromatograms, the negative-ion ESI-MS spectrum, and the UV spectrum for **5** are displayed in Figure 1. The HPLC chromatograms and the ESI-MS spectrum demonstrate the high purity of the preparation and confirm the correct molecular weight. The $[M-H]^-$ peak at m/z = 344.1 agrees well with the calculated exact mass of 344.0653. The UV absorption spectrum is characteristic for the etheno adduct with a shoulder at 215 nm and a well-defined peak at 275 nm.

The ^{1}H NMR data (chemical shifts and scalar couplings) are summarized in Table 1, and the raw data are presented in Supporting Information. The 2′-deoxy-β-D-ribose-3′-phosphate moiety is confirmed on the basis of the shifts and couplings, which are very similar to those reported for the unmodified nucleotide dCyd-3′-*P*.[20] Furthermore, the proton coupling pattern indicates that the conformational equilibrium of the deoxyribose ring between primarily the *S*-type (2′-*endo*, ^{2}T$_3$) and *N*-type (3′-*endo*, ^{3}T$_2$) forms exhibits an *S*/*N* ratio of ca. 1.8 ($J_{1'2'}/J_{3'4'}$), which is typical for deoxyribose pyrimidine nucleotides in the *anti* conformation.[15] The parameters $J_{2'3'}$ = 6.8 Hz and $J_{1'2'} + J_{3'4'}$ = 10.6 Hz are also consistent with the mean values (6.6 and 10.9) found for a series of pyrimidine 3′-nucleotides in which the base adopts primarily the *anti* conformation (as shown in Scheme 1).

The modified base, i.e., the 3,N^4-etheno-5-methyl-cytosine moiety, is confirmed on the basis of the mutually coupled H7

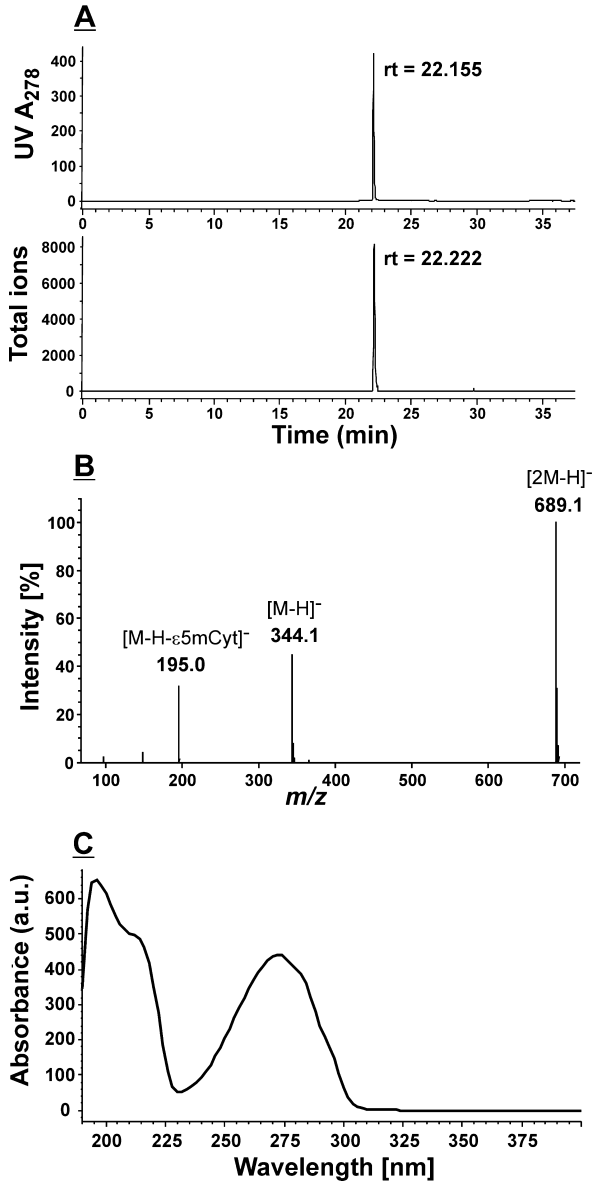

Figure 1. LC-MS and UV characteristics of ε5mdCyd-3′-*P* (**5**). (A) The LC-MS profiles for total-ion current and UV absorption at 278 nm show retention times of ca. 22.2 min for the HPLC conditions described in Experimental Procedures. (B) The negative-ion ESI-MS spectrum confirms the expected molecular weight of 345 and displays a fragment ion derived by neutral loss of the modified base 3,N^4-etheno-5-methyl-cytosine (ε5mCyt). (C) The UV absorption spectrum (in water/acetonitrile) shows the characteristic peak for the adduct at ca. 275 nm.

and 8-Me groups (^4J = 1.26 Hz) and the presence of the etheno protons H2 and H3, which exhibit a pair of doublets with $^3J_{2,3}$ = 2.0 Hz, analogous to data reported for etheno-cytosine (εCyt), etheno-cytidine (εCyd), and derivatives[21] as well as for etheno-2′-deoxycytidine (εdCyd) and derivatives.[19] The assignment of H3 as being downfield of H2 for both N^1-protonated and unprotonated forms was made by chemical means by Barrio et al.[21] as a revision of earlier work and is consistent with the expected deshielding due to the proximity of H3 to C5═O in the ring plane. The reverse assignment, H2 as downfield of H3, for εdCyd was made by Zhang et al.[19] without explanation.

Identification and Chromatographic Characteristics of Nucleoside Adducts. When calf thymus DNA was treated

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with CAA or arachidonic acid-derived LPO products, etheno-adducts of nucleoside residues were formed. Following DNA digestion, the resulting nucleoside-3′-phosphates were converted to ^{32}P-labeled 5′-phosphates as described in the Experimental Procedures section. The various normal and modified nucleotides can be separated using 2D-TLC. For example, three distinct adducts spots were usually found in the upper-left-hand corner of the TLC chromatogram (autoradiograph in Figure 2A). Spots 1 and 2 had been previously and

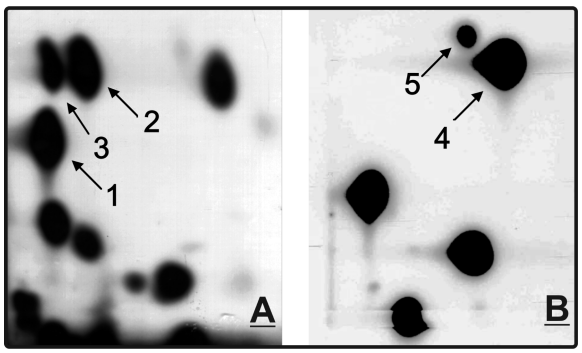

Figure 2. Chromatographic characteristics (2D-TLC) of etheno-nucleotide adducts (A) and unmodified nucleotides (B) obtained from DNA digests using the ^{32}P-postlabeling technique. Calf thymus DNA was treated *in vitro* with adduct-forming compounds as described in the text. The nucleoside-3′-phosphates obtained from digests were converted to ^{32}P-labeled 5′-phosphates as described in the Experimental Procedures. The numbered autoradiographic spots correspond to (1) 1,N^6-etheno-2′-deoxyadenosine-5′-[^{32}P]phosphate (ε*d*Ado-5′-*P**); (2) 3,N^4-etheno-2′-deoxycytidine-5′-[^{32}P]phosphate (ε*d*Cyd-5′-*P**); (3) 3,N^4-etheno-5-methyl-2′-deoxycytidine-5′-[^{32}P]phosphate (ε5mdCyd-5′-*P**, 6); (4) unmodified 2′-deoxycytidine-5′-[^{32}P]-phosphate (dCyd-5′-*P**); and (5) 5-methyl-2′-deoxycytidine-5′-[^{32}P]-phosphate (5mdCyd-5′-*P**) corresponding to compound **4** in Scheme 1.

unequivocally identified as 1,N^6-etheno-2′-deoxyadenosine-5′-[^{32}P]phosphate (ε*d*Ado-5′-*P**) and 3,N^4-etheno-2′-deoxycytidine-5′-[^{32}P]phosphate (ε*d*Cyd-5′-*P**), respectively,[16] while spot 3 was unknown. By chromatographic comparison with our newly synthesized and labeled standard, compound **6**, we were able to identify spot 3 as 3,N^4-etheno-5-methyl-2′-deoxycytidine-5′-[^{32}P]phosphate (ε5mdCyd-5′-*P**).

For quantitative analysis and comparisons, nucleoside adduct levels in DNA can be expressed relative to the total number of residues or to the number of unmodified parent nucleoside residues. As described in Experimental Procedures, the normal nucleotides in a DNA digest were quantitated by HPLC methods. The number of unmodified 5mdC residues was determined by ^{32}P-postlabeling of a highly diluted fraction of the DNA digest followed by 2D-TLC. In Figure 2B, the small spot 5 in the upper-right-hand corner of the TLC chromatogram could be assigned to 5-methyl-2′-deoxycytidine-5′-[^{32}P]-phosphate (5mdCyd-5′-*P**), while the larger spot 4 corresponds to normal 2′-deoxycytidine-5′-[^{32}P]phosphate (dCyd-5′-*P**).

Quantitation of Etheno-Nucleoside Residues in DNA Treated with Adduct-Forming Agents *in Vitro*. The ^{32}P-postlabeling technique was used to determine the levels of three etheno-nucleoside residues (ε5mdC, ε*d*C, and ε*d*A) in untreated calf thymus DNA and after its exposure to 2 mM and 20 mM chloroacetaldehyde (CAA). The results are summarized in the upper part of Table 2 and are expressed as the number of adduct residues per 10^9 residues of the parent nucleoside type or per 10^9 total nucleoside residues (ppb). For the DNA used, methylated deoxycytidine (5mdC) represented 4.7% of the total deoxycytidine content. When nucleoside adduct frequencies were expressed relative to total nucleoside residues, the ratio ε5mdC/ε*d*C was 0.15 without CAA treatment and ca. 0.33 after CAA treatment. However, when individual adduct levels were expressed relative to the number of the corresponding parent nucleosides, then the ε5mdC/ε*d*C ratio was 3.4 in untreated DNA and increased to ca. 7 after CAA exposure. These results prove that methylation of deoxycytidine at the C5 position results in a higher frequency of etheno adduct formation, most likely due to the higher nucleophilicity of the N3 position compared to normal deoxycytidine.

The etheno-deoxyadenosine adduct ε*d*A was also quantified, and its levels were similar to those of ε5mdC when evaluated on the basis of total nucleoside residues. However, on the basis of parent nucleosides, ε*d*A levels were significantly lower than those observed for ε*d*C and ε5mdC, with or without CAA treatment (ε5mdC/ε*d*A ratios in the range 15 to 55). A clearly positive dose−response relationship for CAA treatment was observed for all three etheno adducts.

Lipid peroxidation (LPO) of arachidonic acid (AA) in the presence of ferrous sulfate and NADPH results in the production of reactive agents, such as 4-HNE, which can generate *in situ* in native DNA the same three etheno-nucleoside adducts described above, including the newly identified ε5mdC. The data summarized in the bottom portion of Table 2 demonstrate that *in vitro* incubations of LPO products with calf thymus DNA result in the formation of etheno adducts in a dose-dependent manner. For AA concentrations of 0, 0.25, and 0.5 mM, the ε5mdC/ε*d*C ratio was nearly constant at ca. 0.15, based on total nucleoside residues, or ca. 3.2, based on parent nucleosides. The corresponding ε5mdC/ε*d*A ratios *decreased* from 2.8 to 1.8 or from 91 to 51, respectively, with increasing AA concentration.

Statistical analysis of the results in Table 2 demonstrates that the levels of the three described adducts formed during *in vitro* DNA incubations exhibit strong linear correlations with the following coefficients ($p < 0.05$): ε*d*C vs ε5mdC, $r = 0.98$; ε*d*A vs ε5mdC, $r = 0.97$; ε*d*A vs ε*d*C, $r = 0.94$. These correlations suggest that closely related mechanisms are involved in the formation of these adducts.

Levels of ε5mdC in DNA from Human Tissue and White Blood Cells. Three types of human samples (stored in the Biobank of the German Cancer Research Center, DKFZ), namely, white blood cells ($n = 2$), liver ($n = 2$), and lung tissue ($n = 10$), had been previously analyzed for ε*d*A and ε*d*C levels in DNA. These samples were reanalyzed using our ultrasensitive ^{32}P-postlabeling method, and ε5mdC was unequivocally detected in all samples. The number of ε5mdC residues per 10^9 parent nucleosides were as follows: 65 and 78 in two asymptomatic human liver samples; 6415 and 9468 in two samples of white blood cells from women on a diet high in ω-6 polyunsaturated fatty acid (PUFA, linoleic acid); 31−1865 in ten nontumorous lung specimens from lung cancer patients (mean ± SEM: 397 ± 19). The frequency of the etheno adduct ε5mdC in DNA from human lung tissue was about 10 times higher than that for ε*d*C.

For the 14 samples studied, a statistically significant positive correlation was observed between the formation of ε*d*C and

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ε5mdC (r = 0.97, p < 0.01, Figure 3). A weaker but significant correlation was also found for εdA and ε5mdC (r = 0.79, p < 0.01, data not shown). The correlations observed for these three DNA lesions suggest that they are formed by a common pathway *in vivo*, i.e., via a LPO product such as 4-HNE. This conclusion is further supported by the strong correlations found for *in vitro* adduct formation (Table 2). The steady-state levels of these adducts in DNA *in vivo* are determined by the rates of their formation and their removal by DNA repair processes. While distinct repair pathways and kinetics have been described for εdA and εdC residues,[22] nothing is currently known about the removal of ε5mdC from DNA. Studies are now warranted to elucidate the possible role for this lesion in miscoding and/or perturbation of methylation patterns. Our data established that the etheno-adduct on 5mdC should be added to the known major endogenous DNA damage products.

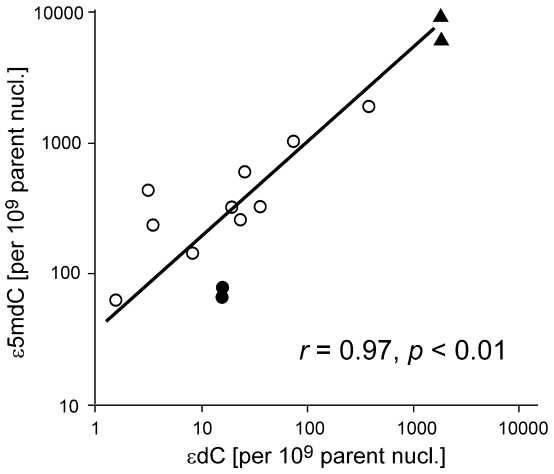

Figure 3. Correlation between the formation of the etheno adduct residues ε5mdC and εdC in human DNA *in vivo*. Numbers of adduct residues per 10^9 parent residues are plotted on a log–log scale for (▲) WBC of female volunteers on a high ω-6 polyunsaturated fatty (linoleic) acid diet (n = 2); (●) asymptomatic human liver tissue biopsies (n = 2); and (○) nontumorous surgical lung specimens from nonsmoking lung cancer patients (n = 10). For all 14 samples, the correlation coefficient is r = 0.97 (p < 0.01).

DISCUSSION

A change in the methylation patterns of genomic DNA is a hallmark for many types of disease, and both hyper- and hypomethylation have been observed.[2] The processes that lead to these changes have not yet been fully elucidated, but there is evidence that DNA damage may play a role.[9] DNA damage in dCpdG sequences disturbs the proper methylation of cytidine in the newly synthesized strands.[9] In fact, dCpdG sequences have been found to be mutational hotspots for several carcinogens, including endogenous carcinogens resulting from oxidative stress and lipid peroxidation, which in turn lead to the formation of the etheno adduct residues εdA and εdC in DNA.[23] These adducts have been consistently detected at background levels in normal DNA, but their frequencies are increased several fold in human and animal tissues in which tumors subsequently develop.[24] These adducts have also been detected in atherosclerotic lesions.[25] The etheno adducts are highly pro-mutagenic lesions,[26] and mutations at dCpdG sites by chloroacetaldehyde (a compound that specifically forms etheno-DNA adducts) were found in one study to be affected by the methylation status of dCpdG islands.[27]

Since etheno adducts can be formed in dCpdG sequences (on dC, 5mdC, and dG residues), one could argue that these types of DNA damage play a role in the alteration of the global methylation status of DNA and, thus, a role in the onset of chronic degenerative diseases. To this end, we synthesized and characterized the new etheno adduct ε5mdCyd-3′-*P* (3,N^4-etheno-5-methyl-2′-deoxycytidine-3′-phosphate), compound **5**, and detected the presence of the corresponding modified ε5mdC residues in tissue DNA and in DNA treated *in vitro* with CAA or LPO-derived products of AA. The frequency of ε5mdC residues was relatively high (6–9 per 10^6 5mdC) in two WBC samples from women on a high ω-6 PUFA diet. In 10 nontumorous lung samples from lung cancer patients, the ε5mdC/εdC ratio was about 10:1. Such "massive" DNA damage could alter the methylation profile in different ways. For instance, the interaction between DNMT and dCpdG islands could be affected, thus altering the methylation rate, or the removal of the damaged base by DNA repair processes would simultaneously remove 5mdC, for instance by (long-patch) nucleotide excision repair, as described for the removal of the 4-HNE-induced etheno-dG adduct.[28]

Etheno adducts (εdC and εdA) have been found to be strongly elevated in human tissues with premalignant conditions related to chronic inflammatory processes, including cirrhosis, ulcerative colitis, Crohn's disease, and familial adenomatous polyposis.[13] These patients are at high risk for developing cancer in the affected organ. Chronic inflammatory processes that occur in hepatocellular cirrhosis and ulcerative colitis are accompanied by marked methylation changes in dCpdG islands in the affected but still nonmalignant tissues.[29] Taken together, these findings support the hypothesis that pro-inflammatory stimuli leading to LPO-derived DNA damage could account, in part, for the epigenetic changes observed during carcinogenesis. In many types of cancer, the expression of DNMT1 was found to be increased,[30] although global hypomethylation was still observed. It is currently not understood why increased DNMT1 expression does not lead to normal methylation levels or even hypermethylation. The presence of ε5mdC residues may reduce the accessibility of DNA for DNMT's and, thus, result in hypomethylation.

In the present work, we did not determine the frequency of ε5mdC in colon tissue from cancer-prone patients. However, εdA and particularly εdC have been found to be significantly increased in subjects suffering from ulcerative colitis, Crohn's disease, and familial adenomatous polyposis.[13] Previously, we observed in DNA digests from colon samples an adduct spot that migrated at the same position as the ε5mdCyd-3′-*P* standard, but our new ultrasensitive detection method was not then available to allow quantitative analysis. Still, from these observations it can be inferred that high levels of ε5mdC are present in colon DNA from these patients. In the future, our ^{32}P-postlabeling method will allow the reliable determination of etheno adduct levels in the DNA obtained from small tissue biopsies.

In colorectal cancer, gene-specific hypermethylation is also often observed, for example, in the gene for the DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT). However, in ulcerative colitis patients prone to colorectal cancer, the promoter hypermethylation of the MGMT gene was significantly less frequent than reported for sporadic colorectal cancers.[31] This suggests that continuous DNA

damage to (methylated) dCpdG sequences by LPO products could occur, leading to hypomethylation, rather than hypermethylation. Thus, one can speculate that the continuous formation and removal of etheno adducts, including ε5mdC, at dCpdG sites could result in a gradual loss of methylation. A link between the repair of etheno adduct residues and DNA methylation processes has already been considered because MBD4 (methyl-dCpdG binding domain protein 4) is involved in the repair of 5-methylcytosine CpG-TpG mismatches.[10] At present, the repair processes that would recognize and remove ε5mdC are still unknown and warrant further investigation. Also, studies on whether etheno adducts influence the coiling and packaging of DNA (e.g., by affecting histone binding) will help to elucidate the complex interaction among methylation, demethylation, adduct formation, and repair.

In conclusion, we show for the first time that inflammation generated lipid peroxidation (LPO) products can react with 5-methyl-2′-deoxycytidine (5mdC) to form ε5mdC *in vitro* and *in vivo*, and therefore, ε5mdC should be added to the list of endogenous types of DNA damage. The availability and characterization of the synthetic reference compounds **4** and **5** and the development of an ultrasensitive and specific assay for the quantitation of ε5mdC lesions in small (ca. 25 μg) human DNA samples provide a novel tool for unraveling the possible role of ε5mdC in the progression of chronic degenerative human diseases.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The details of the synthesis shown in Scheme 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ DEDICATION

We dedicate this work to the late Dr. Jagadeesan Nair.

■ ABBREVIATIONS

ε5mdC, 3,N^4-etheno-5-methyl-2′-deoxycytidine; 5mdC, 5-methyl-2′-deoxycytidine; εdA, 1,N^6-etheno-2′-deoxyadenosine; εdC, 3,N^4-etheno-2′-deoxycytidine; LPO, lipid peroxidation; AA, arachidonic acid; CAA, chloroacetaldehyde; 4-HNE, *trans*-4-hydroxy-2-nonenal

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