(500 MHz) ($CDCl_3$) δ 0.64-1.78 (m, 18 H), 3.43-3.65 (m, 2 H), 3.76 (s, 3 H), 4.54-4.59 (m, 1 H), 6.90-7.88 (m, 9 H). Anal. Calcd for $C_{25}H_{36}BO_3P$: C, 70.43; H, 8.51. Found: C, 70.59; H, 8.48.

(*S*)-*o*-Anisyl(2-hydroxyethyl)phenylphosphine-Borane (19). A solution of **16** (2.13 g, 5 mmol) in dry ether (10 mL) was added to a slurry of $LiAlH_4$ (190 mg, 5 mmol) in ether (10 mL) at 0-10 °C under argon. The mixture was stirred at ambient temperature for 2 h, and the reaction was quenched with diluted HCl. The product was extracted with ether, the combined extracts were washed and dried ($MgSO_4$), and the solvent was evaporated. The residual oil was purified by column chromatography on silica gel eluting with AcOEt/hexane (1:1) to give **19** as a colorless oil (1.20 g, 87%). Enantiomeric excess of this compound was determined to be 100% by HPLC analysis with "CHIRALPAK OP": $[\alpha]^{25}_D$ +4.6° (*c* 1.0, $ClCH_2CH_2Cl$);[40] IR (neat) 3360, 2930, 2370, 1590, 1480, 1255 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.2 (br s, 1 H), 2.5-3.0 (m, 2 H), 3.58 (s, 3 H), 3.6-4.0 (m, 2 H), 6.6-7.9 (m, 9 H). Anal. Calcd for $C_{15}H_{20}BO_2P$: C, 65.73; H, 7.35. Found: C, 65.70; H, 7.53.

(*S*)-*o*-Anisyl-(2-iodoethyl)phenylphosphine-Borane (20). Methanesulfonyl chloride (0.40 g, 3.5 mmol) was added to a solution of **19** (0.79 g, 2.9 mmol) in dry pyridine (1 mL) at 0 °C, and the mixture was stirred at this temperature for 2 h. Water was added, and the product was extracted with ether. The combined extracts were washed with diluted HCl, brine, aqueous $NaHCO_3$, and brine and dried ($MgSO_4$). The solvent was evaporated, and the residue was chromatographed on silica gel with AcOEt/hexane (1:1) as the eluent to give the corresponding methanesulfonate as a pasty oil (0.98 g, 96%). This product was stirred with NaI (8 mmol) in dry acetone (8 mL) at 40 °C for 20 h. Water was added, and the product was extracted with ether. The combined extracts were washed with brine and dried ($MgSO_4$), and the solvent was evaporated. The residue was chromatographed on silica gel with AcOEt/hexane (1:5) to afford **20** (0.79 g, 71% from **19**). Recrystallization from hexane provided a sample for elemental analysis: mp 74-75 °C; $[\alpha]^{25}_D$ +6.7° (*c* 1.0, C_6H_6); IR (KBr) 2350, 1580, 1475, 1240, 1055 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.80-3.40 (m, 4 H), 3.74 (s, 3 H), 6.8-8.0 (m, 9 H). Anal. Calcd for $C_{15}H_{19}BIOP$: C, 46.92; H, 4.99. Found: C, 46.99; H, 5.03.

(*S,S*)-1,4-Bis(*o*-anisylboranatophenylphosphino)butane (21). A solution of activated copper (4 mg atom) in dry THF was prepared according to the Rieke method,[32] and it was cooled to 0 °C. To this solution, **20** (384 mg, 1 mmol) was added in one portion with vigorous stirring. After 30 min, the reaction mixture was passed through a short column of silica gel eluting with CH_2Cl_2. The filtrate was concentrated in vacuo, and the residue was subjected to preparative TLC with AcOEt/hexane (1:2) as the developing solvent to give **21** (51-70%):[41] mp 156-158 °C; $[\alpha]^{25}_D$ +11.8° (*c* 1.0, C_6H_6); IR (KBr) 2350, 1580, 1475, 1250, 1065 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.2-1.9 (m, 4 H), 1.9-2.6 (m, 4 H), 3.58 (s, 6 H), 6.65-7.90 (m, 18 H); ^{31}P NMR (121 MHz) ($CDCl_3$) δ (relative to external $(PhO)_3PO$) 25.2 (br s), 25.8 (br s); ^{11}B NMR (96 MHz) ($CDCl_3$) δ (relative to external $(CH_3O)_3B$) -57.3 (br s). Anal. Calcd for $C_{30}H_{38}B_2O_2P_2$: C, 70.08; H, 7.45. Found: C, 69.73; H, 7.35.

(*S,S*)-1,4-Bis(*o*-anisylphenylphosphino)butane (22). Bis(phosphine-borane) **21** (200 mg, 0.39 mmol) was dissolved in degassed morpholine (2 mL) under argon, and the solution was kept at 70 °C for 2 h. The solvent was removed in vacuo, and the residue was subjected to preparative TLC (benzene) under argon to give **22** (161 mg, 85%): mp 99.0-101.5 °C; $[\alpha]^{25}_D$ -18.2° (*c* 1.0, C_6H_6); IR (KBr) 2920, 1570, 1460, 1435, 1245, 1025 cm^{-1}; ^1H NMR (C_6D_6) δ 1.59-1.70 (m, 4 H), 1.88-1.94 (m, 2 H), 2.08-2.12 (m, 2 H), 3.18 (s, 6 H), 6.43-7.54 (m, 18 H); ^{31}P NMR (121 MHz) ($CDCl_3$) δ (relative to external $(PhO)_3PO$) 58.1 (s), 58.3 (s); HRMS calcd for $C_{30}H_{32}O_2P_2$ 486.1880, found 486.1886.

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Specific Detection of C-4′ Hydroxylated Abasic Sites Generated by Bleomycin and Neocarzinostatin in DNA

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Abstract: A new, general method for detection and quantitation of C-4′ hydroxylated abasic sites generated by bleomycin and neocarzinostatin in DNA was described. The specific detection method was based on a chemical and enzymatic transformation of C-4′ hydroxylated abasic sites to deoxynucleoside 3′-(3-pyridazinylmethyl) phosphates. Enzymatic digestion of 3′-(3-pyridazinylmethyl) 2′-deoxycytidylyl-(3′-5′)-2′-deoxy-3′-guanylate (**7**) with snake venom phosphodiesterase (s.v. PDE) and alkaline phosphatase (AP) gave 2′-deoxycytidine and 3′-(3-pyridazinylmethyl) 2′-deoxyguanylate (**4**) in high yields, indicating that a pyridazine-substituted phosphodiester bond at the 3′-end resists digestion with s.v. PDE. Enzymatic digestion of d(CGCGAATTCGCG) treated with photoactivated green cobalt-peplomycin (Co·PEM) with s.v. PDE and AP following treatment with aqueous hydrazine was examined. Consistent with the previous results on the cleavage of this dodecanucleotide, **4** was obtained as a major product. Photoactivated Co·PEM also mediated spontaneous thymine release from poly(dA-dT) with formation of **5** and **6**. Digestion of Co·PEM-treated calf thymus DNA having a C-4′ hydroxylated abasic site gave pyridazine derivatives **3**-**6** and the total amount of **3**-**6** corresponded well to the sum of spontaneously released free bases. Also investigated was the formation of **3**-**6** in Fe·PEM- and neocarzinostatin- (NCS-) mediated degradation of calf thymus DNA. Hydrazine treatment of their reaction mixtures followed by enzymatic digestion produced pyridazine derivatives **3**-**6**, indicating that C-4′ hydroxylated abasic sites are actually produced in calf thymus DNA. Quantitative analysis indicated that C-4′ hydroxylation is estimated to be a minimum of 17% of the total event caused by the action of NCS on calf thymus DNA.

The chemistry of DNA backbone oxidation mediated by naturally occurring antitumor antibiotics[1] and designed DNA-cleaving molecules[2] is a topic of intense current interest. Such DNA cleavers usually oxidize the deoxyribose moiety via hydrogen

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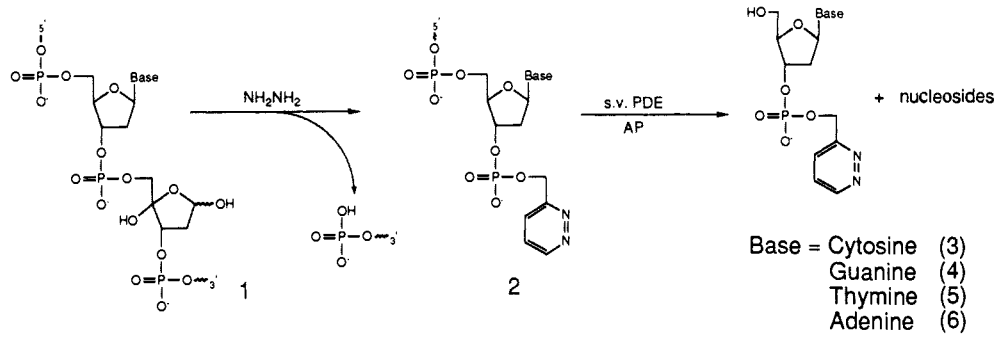
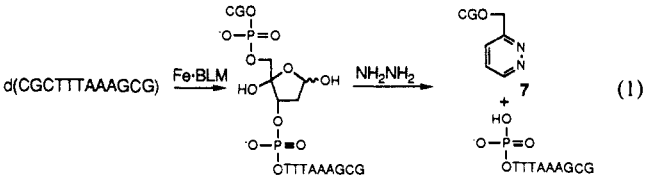
Scheme I



abstraction followed by incorporation of an oxygen functionality onto the deoxyribose moiety, ultimately producing strand breakage and the alkaline-labile lesion that accompanies free base release.[1a,c,2e,3] While the structure of alkaline-labile product resulting from ribose C-4′ hydroxylation has recently been established in bleomycin- (BLM-) mediated degradation of oligonucleotides,[4] no general method for the specific detection of such C-4′ hydroxylated abasic sites (**1**) formed in longer DNA is available. We describe a general method for specific detection and quantitation of such abasic sites in DNA based on a chemical and enzymatic transformation to deoxynucleoside 3′-(3-pyridazinylmethyl) phosphates **3**–**6** as outlined in Scheme I. By using this technique, we were able to detect previously unobserved C-4′ hydroxylated abasic sites in neocarzinostatin- (NCS-) mediated degradation of calf thymus DNA.[1c,5]

Results and Discussion

Enzymatic Digestion of Oligonucleotides Containing 3′-Pyridazinylmethyl Termini. It has been demonstrated that treatment of the Fe·BLM-derived alkaline-labile site of d-($5'$CGCTTTAAAGCG) with hot aqueous hydrazine induces strand cleavage with formation of 3′-(3-pyridazinylmethyl) 2′-deoxycytidylyl-(3′–5′)-2′-deoxy-3′-guanylate (**7**).[4a] Since the



(1)

transformation to pyridazine **7** accompanying strand scission is specific and efficient,[4a,5b] we have employed this hydrazine reaction for the assay of C-4′ hydroxylated abasic sites. For a reliable and accurate assay, specific and quantitative enzymatic digestion of modified DNA fragments possessing the pyridazinylmethyl group at their 3′-termini, such as **2**, to pyridazine derivatives **3**–**6** is indispensable.[6] We therefore prepared pyridazine dinucleotide

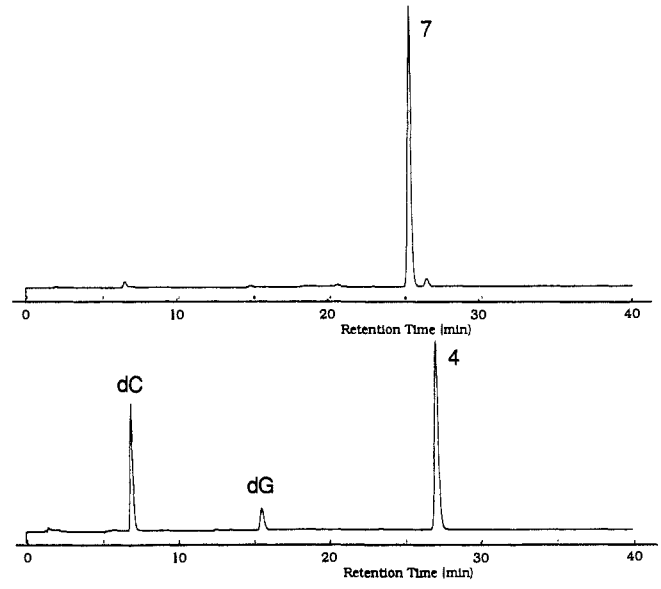
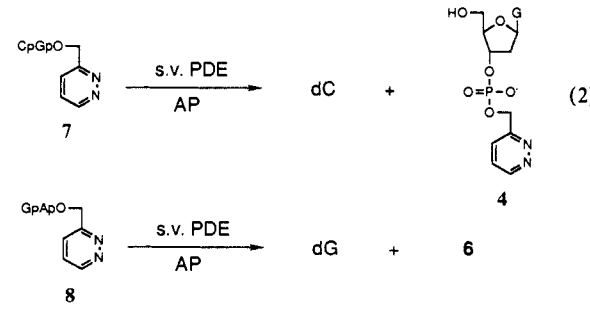


Figure 1. HPLC profiles of **7** (a, top) before enzymatic digestion and (b, bottom) after enzymatic digestion with snake venom phosphodiesterase and calf intestine alkaline phosphatase. The peaks indicated comigrated with authentic samples. Analysis was carried out on a Cosmosil $5C_{18}$ column (4.6 × 150 mm); elution was with 0.05 M ammonium formate containing a 0–8% acetonitrile linear gradient, 40 min, at a flow rate of 1.5 mL/min.

7 according to the published procedure[4a] and examined its behavior toward enzymatic digestion with phosphodiesterase of different sources. When dinucleotide **7** was incubated with snake venom phosphodiesterase (s.v. PDE) and alkaline phosphatase (AP) at 37 °C for 2 h in 50 mM Tris-HCl buffer (pH 7.2), both dC and **4** were obtained in high yields (Figure 1), whereas calf spleen phosphodiesterase digested **7** quantitatively to dC and dG, showing that the pyridazine-substituted phosphodiester bond at the 3′-end resists digestion with s.v. PDE. An analogous pyridazine dinucleotide, 3′-(3-pyridazinylmethyl) 2′-deoxyguanylyl-(3′–5′)-2′-deoxy-3′-adenylate (**8**), was also digested to dG and **6** in high yields under similar conditions. Essentially the same results were obtained in prolonged incubation in both cases. These results strongly suggest that under the usual digestion conditions, s.v. PDE hydrolyzes a normal phosphodiester bond efficiently but does not digest the phosphodiester bond at the 3′-pyridazinylmethyl end.



(2)

(1) (a) Hecht, S. M. *Acc. Chem. Res.* **1986**, *19*, 83. (b) Hurley, L. H.; Needham-VanDevanter, D. R. *Ibid.* **1986**, *19*, 230. (c) Goldberg, I. H. *Free Radical Biol. Med.* **1987**, *3*, 41, and references therein. (d) Chrisey, L. A.; Shahidi Bonjar, G. H.; Hecht, S. M. *J. Am. Chem. Soc.* **1988**, *110*, 644, and references therein. (e) Zein, N.; Sinha, A.; McGahren, W. J.; Ellestad, G. A. *Science* **1988**, *240*, 1198. (f) Long, B. H.; Golik, J.; Forenza, S.; Ward, B.; Rehfuss, R.; Dabrowiak, J. C.; Catino, J. J.; Musial, S. T.; Brookshire, K. W.; Doyle, T. W. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *186*, 2.

(2) (a) Dervan, P. B. *Nucleic Acids Mol. Biol.* **1988**, *2*, 49. (b) Dervan, P. B. *Science* **1986**, *232*, 464. (c) Barton, J. K. *Ibid.* **1986**, *233*, 727. (d) Fleisher, M. B.; Mei, H.-Y.; Barton, J. K. *Nucleic Acids Mol. Biol.* **1988**, *2*, 65. (e) Sigman, D. S. *Acc. Chem. Res.* **1986**, *19*, 180. (f) Lown, L. W.; Joshua, A. V. *J. Chem. Soc., Chem. Commun.* **1982**, 1298.

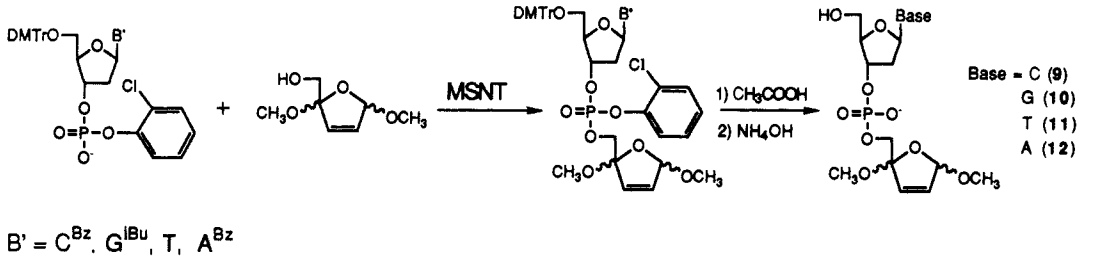
(3) Stubbe, J.; Kozarich, J. W. *Chem. Rev.* **1987**, *87*, 1107.

(4) (a) Sugiyama, H.; Xu, C.; Murugesan, N.; Hecht, S. M.; van der Martel, G. A.; van Boom, J. H. *Biochemistry* **1988**, *27*, 58. (b) Rabow, L.; Stubbe, J.; Kozarich, J. W.; Gerlt, J. A. *J. Am. Chem. Soc.* **1986**, *108*, 7130.

(5) (a) C-1′ hydrogen abstraction giving 2′-deoxyribonolactone; see: Kappen, L. S.; Goldberg, I. H. *Biochemistry* **1989**, *28*, 1027. (b) Saito, I.; Kawabata, H.; Fujiwara, T.; Sugiyama, H.; Matsuura, T. *J. Am. Chem. Soc.* **1989**, *111*, 8302.

(6) An analogous enzymatic digestion of Fe·BLM-treated calf thymus DNA with λ exonuclease; see: Murugesan, N.; Xu, C.; Ehrenfeld, G. M.; Sugiyama, H.; Kilkuskie, R. E.; Rodriquez, L. O.; Hecht, S. M. *Biochemistry* **1985**, *24*, 5735.

Scheme II



B' = C^{Bz}, G^{iBu}, T, A^{Bz}

Table I. Quantitative Analysis of Products Formed in Peplomycin-Mediated Degradation of d(CGCGAATTCGCG), Poly(dA-dT), and Calf Thymus DNA

		free base, μM					pyridazine,[a] μM				
	substrate	C	G	T	A	total	3	4	5	6	total
Co·PEM	d(CGCGA_2T_2CGCG)	33.2	0.2	4.2	3.4	40.8	0	37.1	4.4	2.3	43.8
Co·PEM	poly(dA-dT)			43.2	3.7	46.9			3.0	40.0	43.0
Co·PEM	calf thymus	19.8	6.0	27.0	16.8	69.6	2.3	39.7	6.1	12.6	60.7
Fe·PEM	calf thymus	14.9	6.8	14.0	16.3	52.0	3.4	38.3	3.6	9.7	55.0

[a] Quantitated as corresponding nucleosides after treatment with snake venom phosphodiesterase and AP by reverse-phase HPLC.

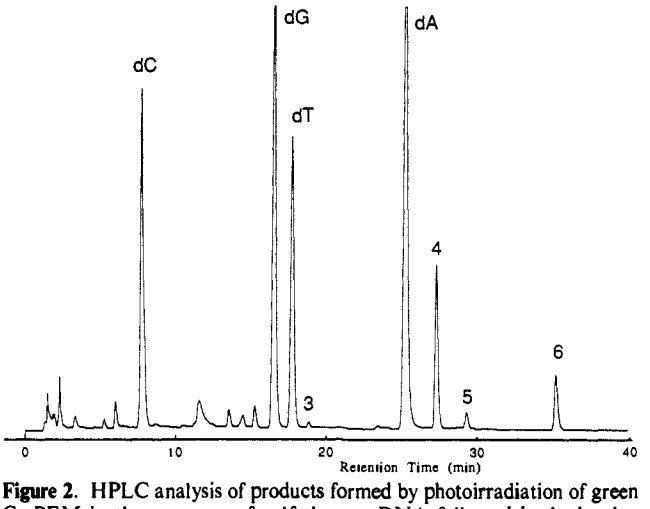
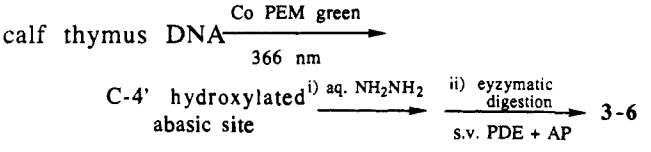


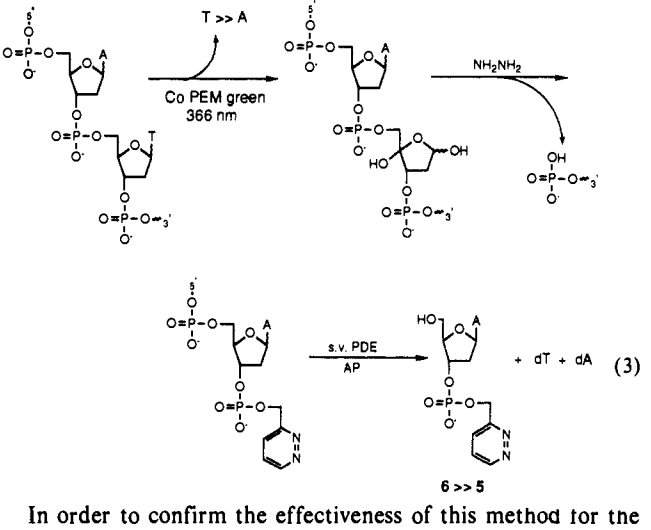
Figure 2. HPLC analysis of products formed by photoirradiation of green Co·PEM in the presence of calf thymus DNA followed by hydrazine treatment and subsequent enzymatic digestion with snake venom phosphodiesterase and calf intestine alkaline phosphatase. The peaks indicated comigrated with authentic samples. Analysis was carried out on a Cosmosil $5C_{18}$ column (4.6 × 150 mm); elution was with 0.05 M ammonium formate containing a 0–8% acetonitrile linear gradient, 40 min, at a flow rate of 1.5 mL/min.

Thus, four possible pyridazine derivatives **3–6** were prepared by the route shown in Scheme II, and their stabilities against s.v. PDE digestion were examined. Under the standard conditions, **3** and **5** were quite stable, whereas only small amounts of purine nucleotides **4** and **6** were hydrolyzed to dG and dA, respectively. These results suggest that s.v. PDE digestion of modified DNA fragments like **2** would produce **3–6** almost quantitatively under appropriate conditions and strongly encouraged us to develop a new, specific assay method for C-4′ hydroxylated abasic sites in longer DNA.

Detection of Pyridazine Mononucleotide from Co·PEM-Treated d(CGCGAATTCGCG), Poly(dA-dT), and Calf Thymus DNA. The photoactivated green cobalt complex of peplomycin (PEM), a derivative of BLM, has been demonstrated to induce ribose C-4′ hydroxylation preferentially at GC and GT sites.[7] We first examined the enzymatic digestion of d(CGCGAATTCGCG) modified by photoactivated green Co·PEM[7e,8] with s.v. PDE and AP following treatment with aqueous hydrazine, since Co·PEM has been shown to generate a C-4′ hydroxy abasic site at two GC sites in this dodecanucleotide. As expected, **4** was obtained as a major product and the total amount of **3–6** agreed well with the sum of spontaneously released free base (Table I). Photoactivated green Co·PEM also mediated the release of thymine and adenine from poly(dA-dT). Following hydrazine treatment and subsequent digestion with s.v. PDE and AP of the mixture, reasonable amounts of **5** and **6** were produced, showing that the C-4′ hydroxylated abasic sites of poly(dA-dT) also produce **5** and **6** quite efficiently. The amounts of **5** and **6** corresponded well to those of spontaneously released adenine and thymine, respectively, showing again the reliability of this assay method.

Scheme III





In order to confirm the effectiveness of this method for the quantitation of the C-4′ hydroxylated abasic site in a longer DNA substrate, enzymatic digestion of Co·PEM-treated calf thymus DNA having a C-4′ hydroxylated abasic site was carried out. As shown in Figure 2 formation of **3–6** was clearly observed on reverse-phase HPLC together with four nucleosides. The peaks

(7) (a) Chang, C.-H.; Meares, C. F. *Biochemistry* **1982**, *21*, 6332. (b) Chang, C.-H.; Meares, C. F. *Ibid.* **1985**, *24*, 3060. (c) Subramanian, R.; Meares, C. F. *J. Am. Chem. Soc.* **1986**, *108*, 6427. (d) Morii, T.; Saito, I.; Matsuura, T.: Kuwahara, J.: Sugiura, Y. *Ibid.* **1987**, *109*, 938. (e) Saito, I.; Morii, T.; Sugiyama, H.; Matsuura, T.; Meares, C. F.; Hecht, S. M. *Ibid.* **1989**, *111*, 2307.

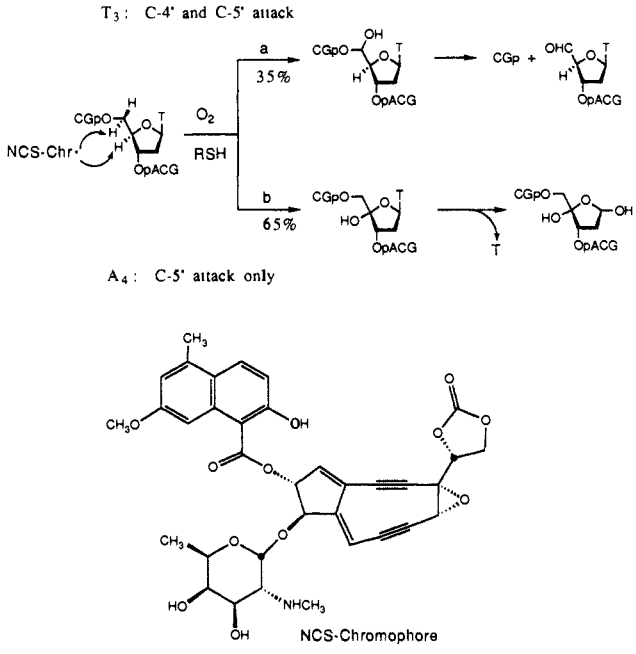
(8) Chang, C.-H.; Dallas, J. L.; Meares, C. F. *Biochem. Biophys. Res. Commun.* **1983**, *110*, 959.

Table II. Quantitative Analysis of Products Formed in Neocarzinostatin-Mediated Degradation of d(CGATCG) and Calf Thymus DNA

substrate	OH^- treatment	free base, μM					pyridazine,[a] μM				
		C	G	T	A	total	3	4	5	6	total
d(CGTACG)	*b*		1.4	7.3	1.0	9.7	0	8.2	0	0	8.2
d(CGTACG)	*c*		2.0	10.0	22.2	34.2					
calf thymus	*b*		1.3	5.6	2.8	9.7	2.2	1.4	0.8	0.6	5.0
calf thymus	*c*	*d*	*d*	20.9	8.3	29.2					

[a] Quantitated as corresponding nucleosides after treatment with snake venom phosphodiesterase and AP by reverse-phase HPLC. [b] Determined without alkal treatment. [c] After alkali treatment (0.5 N NaOH, 90 °C), released free bases were determined by HPLC. [d] Not determined due to the overlap of the peak to other peaks.

Scheme IV



corresponding to **3–6** were collected and identified by comparison of their HPLC behaviors in two solvent systems and of their UV spectral data with those of authentic samples. As listed in Table I, the major product was a pyridazine derivative of dG (**4**), which amounted to 65% of the total pyridazines. These results are entirely consistent with the reported sequence specificity of Co·BLM wherein 4′-hydroxylated abasic sites are formed preferentially at the 3′-sites of guanine moiety.[7b,d] Moreover, the total amount of **3–6** corresponded well to the sum of spontaneously released free bases. These results indicate that the present method using hydrazine can be used as a reliable and considerably accurate assay of C-4′ hydroxylated abasic sites formed in DNA. In control experiments, we confirmed that hydrazine treatment never induces modification of calf thymus DNA, oligonucleotides, or pyridazine mononucleotides **3–6**.

Formation of C-4′ hydroxylated abasic sites was also detected in Fe(II)·PEM·O_2-mediated modification of calf thymus DNA,[4a] where again the total amount of pyridazine derivatives is equal to the sum of spontaneously released free bases. It has been known that Fe(II)·PEM·O_2 induces strand cleavage with the formation of glycolic acid termini at the 3′-end.[9] Present results indicate that this assay method for detection of C-4′ hydroxylated abasic sites is equally effective even in the presence of other type of DNA lesion.

Neocarzinostatin-Induced C-4′ Hydroxylated Abasic Sites in d(CGTACG) and Calf Thymus DNA. We previously observed that a significant amount of C-4′ hydroxylation occurs at thymine-3 (T_3) of self-complementary hexanucleotide d-($^5C_1G_2T_3A_4C_5G_6$) in competition with C-5′ oxidation at adenine-4

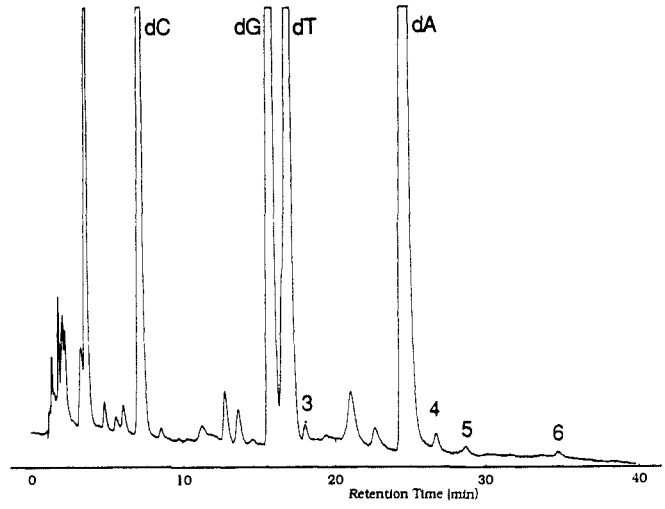


Figure 3. HPLC analysis of products formed from NCS-treated calf thymus DNA followed by hydrazine treatment and subsequent enzymatic digestion with snake venom phosphodiesterase and calf intestine alkaline phosphatase. The peaks indicated comigrated with authentic samples. Analysis was carried out as indicated in Figure 2.

(A_4) (Scheme IV).[5b] In fact, formation of **4** was confirmed by this assay method as shown in Table II. In order to know whether such C-4′ hydroxylation actually occurs in longer DNA substrates, we examined the formation of **3–6** from NCS-treated calf thymus DNA.[1c,5,10] Since it has recently been reported that C-1′ oxidation occurs at a d(AGC) sequence,[11] it is very important to evaluate the contribution of the C-4′ hydroxylation pathway to the total event induced by NCS.

When calf thymus DNA (1 mM, base concentration) was incubated for 12 h with NCS (250 μM) in the presence of 4-hydroxythiophenol[10c] (4 mM) as an activator, thymine was released spontaneously in preference to other bases (Table II). Hydrazine treatment of the mixture and subsequent enzymatic digestion produced four pyridazine derivatives, **3–6**, indicating that NCS actually oxidizes ribose C-4′ to produce C-4′ hydroxylated abasic sites in calf thymus DNA (Figure 3).

Spontaneous release of larger amounts of free base relative to pyridazines is due to ribose C-5′ oxidation by NCS.[1c,10c,12] The total event induced by NCS (29.2 μM) was easily accessible by quantitation of the free base released by hot alkali treatment (0.1 M NaOH, 90 °C, 5 min) as shown in Table II.[5b,10c] Therefore, formation of pyridazine derivatives **3–6** (total 5.0 μM) via C-4′ hydroxylation is estimated to be a minimum 17% of the total event (29.2 μM) caused by action of NCS. Thus, the C-4′ hydroxylation path discovered in the modification of self-complementary hexanucleotide d(CGTACG) has now been proven to play a significant role in NCS-mediated degradation of calf thymus DNA as well.

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Among pyridazine derivatives (**3**–**6**) obtained, the amount of adenosine derivative **6** was less than the others. In addition, formation of **6** was not observed in the reaction of d(GCATGC)[10c] or poly(dA-dT), where only C-5′ oxidation[1c,10,12] occurred specifically. These results indicate that an AT sequence may not produce such C-4′ hydroxylated abasic sites. In sharp contrast, a TA sequence may well produce C-4′ hydroxylated abasic sites in competition with normal C-5′ hydrogen abstraction, as actually observed with d(CGTACG).

In conclusion, we have demonstrated a new, general method for detection and quantification of C-4′ hydroxylated abasic sites in DNA. By using this assay method, we were able to uncover the previously unobserved C-4′ hydroxylation pathway in NCS-mediated degradation of calf thymus DNA. We believe that the present method would be highly useful for investigating the DNA lesion induced by other natural or unnatural DNA-damaging compounds. In fact, since the possibility of C-4′ oxidation by calicheamicin has been pointed out,[13] the present method would be very useful to get clear evidence for the formation of such C-4′ hydroxylated abasic sites.

Experimental Section

Materials and Methods. Peplomycin sulfate was obtained from Nippon Kayaku Co., Ltd. through the courtesy of Dr. T. Takita. Neocarzinostatin was obtained from the Pola Kasei Co. Ltd. research and development laboratory. The concentration of NCS was determined spectrophotometrically (ϵ_{340} 10 800 mM^{-1} cm^{-1}).[14] Calf intestine alkaline phosphatase (AP, 1000 units/mL) and snake venom phosphodiesterase (s.v. PDE, 3 units mL) were purchased from Boehringer Mannheim. Calf thymus DNA was purchased from Pharmacia and sonicated to a 150–200 base pair range by a reported procedure;[15] its concentration was determined by complete digestion with s.v. PDE and AP to 2′-deoxymononucleosides. Protected mononucleoside 3′-(*o*-chlorophenyl) phosphates were prepared according to a published procedure.[16] 3′-(3-Pyridazinylmethyl) 2′-deoxycytidylyl-(3′–5′)-2′-deoxy-3′-guanylate (**7**) and 3′-(3-pyridazinylmethyl) 2′-deoxyguanylyl-(3′–5′)-2′-deoxy-3′-adenylate (**8**) were prepared by a reported procedure.[4a] **8** ^{1}H NMR (D_2O, TSP) δ 2.16 (ddd, 1 H, *J* = 13.9, 8.9, 5.3 Hz, G2′), 2.39 (dd, 1 H, *J* = 14.1, 5.9 Hz, A2′), 2.67 (ddd, 1 H, *J* = 13.9, 5.5, 2.4, Hz, G2′), 2.88 (ddd, 1 H, *J* = 14.1, 8.1, 6.4 Hz, A2′), 3.70 (d, 2 H, *J* = 4.0 Hz, 5′), 4.11 (br s, 1 H, 5′), 4.18 (br s, 1 H, 4′), 4.41 (br s, 1 H, 4′), 4.78 (m, 1 H, 3′), 5.08 (br s, 1 H, 4′), 5.26 (d, 2 H, *J* = 8.4 Hz, 3″-CH_2), 5.88 (dd, 1 H, *J* = 8.9, 5.5 Hz, G1′), 6.31 (dd, 1 H, *J* = 8.1, 5.9 Hz, A1′), 7.74 (s, 1 H, G8), 7.85 (ddd, 1 H, *J* = 8.6, 4.9 Hz, 5″), 7.98 (d, 1 H, *J* = 8.6 Hz, 4″), 8.04 (s, 1 H, A2), 8.38 (s, 1 H, A8), 9.09 (d, 1 H, *J* = 4.9 Hz, 6″). 2,4,6-(Trimethylphenylsulfonyl)-3-nitrotriazolide (MSNT) was purchased from Dojindo Laboratories. CM-Sephadex C-25 was purchased from Pharmacia. SEP-PAK C18 cartridges were purchased from Waters. Silica gel column chromatography was carried out on a Wakogel C-200. Preparative TLC was carried out on Merck silica gel 60 F_{254} plates. HPLC analysis was carried out on a YMC AQ 302 $5C_{18}$ (4.6 × 150 mm) or a Cosmosil $5C_{18}$ column (4.6 × 150 mm). ^{1}H NMR spectra were recorded on a JEOL-JNM-GX400 spectrometer.

Preparation of Cobalt–Peplomycin (Co·PEM) Green Complex. Cobalt peplomycin green complex was prepared by a modified procedure of a published method.[8] To a solution (15 mL) of peplomycin sulfate (60 mg, 0.04 mmol) was added 11 mg of $CoCl_2 \cdot 6H_2O$ (0.046 mmol) and the resultant solution was adjusted to pH 7.0 with 1 N NaOH and allowed to stand at room temperature overnight. The resulting greenish brown solution was concentrated and then applied to CM-Sephadex C-25 (20 g, 2.2 cm × 20 cm) and eluted with a 1.2 L of linear gradient (0–1 M NaCl) containing 0.05 M sodium citrate (pH 4.5). Fractions containing each Co·PEM complex were loaded on four SEP-PAK cartridges and washed with 50 mL of distilled water. The complex was eluted with methanol–water (3:2) and concentrated. After lyophilization, 20 mg of Co·PEM green complex and 23 mg of Co·PEM brown complex were obtained. For Co·PEM green complex: ^{1}H NMR (D_2O, TSP) δ 0.68 (d, 3 H, *J* = 6.9 Hz), 0.93 (d, 3 H, *J* = 6.3 Hz), 1.07 (m, 1 H), 1.24 (d, 3 H, *J* = 6.5 Hz), 1.70 (d, 3 H, *J* = 6.6 Hz), 1.95 (m, 2 H), 2.42 (s, 3 H), 2.86 (m, 2 H), 2.96 (m, 2 H), 3.07–3.59 (m, 11 H), 3.71–4.19 (m, 12 H), 4.29 (m, 1 H), 4.41 (d, 2 H, *J* = 3.0 Hz), 4.96 (s, 1 H), 5.04 (d, 1 H, *J* = 3.0 Hz), 5.17 (m, 1 H), 5.37 (d, 1 H, *J* = 4.3 Hz), 5.58 (d, 1 H, *J* = 3.0 Hz), 7.27 (t, 1 H, *J* = 7.4 Hz), 7.34 (t, 2 H, *J* = 7.2 Hz), 7.43 (d, 2 H, *J* = 7.1 Hz), 7.66 (s, 1 H), 7.77 (s, 1 H), 8.05 (s, 1 H), 8.70 (s, 1 H); ^{13}C NMR (D_2O, TSP) δ 10.4, 12.3, 20.4, 21.5, 22.7, 28.6, 35.7, 39.2, 42.2, 43.4, 45.8, 48.3, 49.8, 52.2, 59.6, 60.3, 60.8, 61.2, 63.8, 64.2, 65.5, 67.0, 68.8, 70.3, 70.7, 71.0, 72.1, 72.2, 72.3, 77.2, 78.0, 97.7, 99.0, 120.5, 121.8, 122.7, 127.8, 130.6, 132.4, 132.6, 136.5, 138.5, 144.8, 150.0, 151.5, 153.3, 160.4, 165.4, 166.0, 169.8, 171.8, 173.4, 173.6, 175.1, 177.0, 177.4, 180.3, 181.2. For Co·PEM brown complex: ^{1}H NMR (D_2O, TSP) δ 1.01 (d, 3 H, *J* = 3.0 Hz), 1.02 (d, 3 H, *J* = 2.2 Hz), 1.13 (d, 3 H, *J* = 6.4 Hz), 1.69 (d, 3 H, *J* = 6.8 Hz), 2.02 (m, 3 H), 2.48 (s, 1 H), 2.90 (m, 2 H), 2.98 (m, 2 H), 3.18–3.66 (m, 11 H), 3.73–4.20 (m, 14 H), 4.44 (dd, 1 H, *J* = 6.8, 3.4 Hz), 4.96 (s, 1 H), 5.03 (m, 1 H), 5.35 (d, 1 H, *J* = 3.2 Hz), 5.45 (d, 1 H, *J* = 4.3 Hz), 5.66 (d, 1 H, *J* = 3.2 Hz), 7.29 (t, 1 H, *J* = 7.3 Hz), 7.36 (t, 2 H, *J* = 7.4 Hz), 7.44 (d, 2 H, 7.2 Hz), 7.69 (s, 1 H), 7.96 (s, 1 H), 8.16 (s, 1 H), 8.72 (s, 1 H); ^{13}C NMR (D_2O, TSP) δ 12.0, 14.4, 17.2, 21.3, 21.9, 28.3, 34.9, 35.8, 38.9, 41.9, 45.1, 45.5, 47.8, 50.6, 52.3, 59.4, 59.9, 61.0, 62.2, 63.6, 64.0, 65.6, 66.7, 68.6, 70.0, 70.5, 70.9, 71.6, 72.0, 77.0, 77.2, 77.8, 97.5, 98.7, 121.6, 121.7, 122.6, 128.0, 130.4, 132.1, 132.4, 136.3, 138.2, 144.8, 149.7, 151.5, 152.0, 160.2, 165.4, 166.2, 170.0, 171.3, 172.5, 173.2, 173.6, 174.7, 176.7, 176.8, 181.1.

Synthesis of 2,5-Dimethoxy-2,5-dihydrofurfuryl 2′-Deoxy-3′-cytidylate (9). A mixture of 5′-*O*-(dimethoxytrityl)-*N*-benzoyl-2′-deoxycytidine-3′-(*o*-chlorophenyl) phosphate triethylammonium salt (115 mg, 0.12 mmol) and 2,5-dimethoxy-2,5-dihydrofurfuryl alcohol[17] (30 mg, 0.19 mmol) was evaporated with pyridine (three times) and dissolved in dry pyridine (30 mL). To this solution, (2,4,6-trimethylphenylsulfonyl)-3-nitrotriazolide (MSNT, 56 mg, 0.19 mmol) was added and the solution was stirred for 1 h. After evaporation of the solvent, the residue was extracted with methylene chloride. The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The resulting residue was subjected to preparative TLC (silica gel, 95% methylene chloride–5% methanol). The crude product thus obtained was treated with 80% aqueous acetic acid (50 mL) at room temperature for 30 min, and the solvent was evaporated under reduced pressure. The residue was treated with concentrated ammonium hydroxide–pyridine (10:1, 10 mL) at 55 °C for 12 h and then concentrated. The resulting residue was subjected to preparative TLC (silica gel, 2-propanol–concentrated ammonium hydroxide, 3:2). After purification by reverse-phase HPLC (YMC AQ 302 $5C_{18}$; 0.05 M ammonium formate containing 0–6% acetonitrile, for 20 min; flow rate, 1.5 mL/min; retention time, 11.2 min) and subsequent lyophilization, a white powder of 2,5-dimethoxy-2,5-dihydrofurfuryl 2′-deoxy-3′-cytidylate (**9**) was obtained: yield 8.5 mg (10%); ^{1}H NMR (D_2O, TSP) δ 2.36 (m, Na^+). H, 2′), 2.61 (ddd, 1 H, *J* = 14.2, 6.0, 3.3 Hz, 2′), 3.25 (s, 3 H, OCH_3), 3.54 (m, 3 H, OCH_3), 3.78 (dd, 1 H, *J* = 12.5, 5.2 Hz, 5″), 3.86 (m, 2 H, 5′), 3.99 (dd, 1 H, *J* = 11.5, 5.2 Hz, 5″), 4.24 (m, 1 H, 4′), 4.74 (m, 1 H, 3′), 5.68 (s, 1 H, 1″), 6.16 (d, 1 H, *J* = 6 Hz, 3″), 6.26 (m, 3 H, 1′, 2″, 6), 7.95 (d, 1 H, *J* = 7.6 Hz, 5); SIMS (positive ion) *m*/*z* 450 $(M + 1)^+$, 472 $(M + Na^+)$.

Synthesis of 2,5-Dimethoxy-2,5-dihydrofurfuryl 2′-Deoxy-3′-guanylate (10). A similar procedure for the synthesis of **9** was followed by using 5′-*O*-(dimethoxytrityl)-*N*-isobutyl-2′-deoxyguanosine 3′-(*o*-chlorophenyl) phosphate triethylammonium salt: yield 10%; ^{1}H NMR (D_2O, TSP) δ 2.37 (m, 1 H, 2′), 2.61 (m, 1 H, 2′), 3.25 (s, 3 H, OCH_3), 3.54 (m, 3 H, OCH_3), 3.78 (dd, 1 H, *J* = 11.0, 5.0 Hz, 5′), 3.87 (m, 2 H, 5′, 5″), 3.99 (dd, 1 H, *J* = 11.0, 5.0 Hz, 5″), 4.23 (m, 1 H, 4′), 4.74 (m, 1 H, 3′), 5.68 (s, 1 H, 1″), 6.16 (d, 1 H, *J* = 6.0 Hz, 3″), 6.25–6.31 (m, 3 H, 1′, 2″, 6), 7.95 (d, 1 H, *J* = 6.7 Hz); SIMS (positive ion) *m*/*z* 490 $(M + 1)^+$.

Synthesis of 2,5-Dimethoxy-2,5-dihydrofurfuryl 2-Deoxy-3′-thymidylate (11). A similar procedure for the synthesis of **9** was followed by using 5′-*O*-(dimethoxytrityl)thymidine 3′-(*o*-chlorophenyl) phosphate triethylammonium salt: yield 12%; ^{1}H NMR (D_2O, TSP) δ 1.91 (d, 3 H, *J* = 1.1 Hz, 5-CH_3), 2.42 (ddd, 1 H, *J* = 14.1, 8.2, 6.6 Hz, 2′), 2.54 (ddd, 1 H, *J* = 14.1, 6.6, 3.1 Hz, 2′), 3.26 (s, 3 H, OCH_3), 3.55 (d, 3 H, *J* = 2.2 Hz, OCH_3), 3.80 (dd, 1 H, *J* = 12.7, 4.9 Hz, 5′), 3.87 (m, 2 H, 5′, 5″), 3.99 (m. 1 H, 5″), 4.20 (ddd, 1 H, *J* = 4.9, 3.4, 3.1 Hz, 4′), 4.77 (m, 1 H, 3′), 5.69 (dd, 1 H, *J* = 2.3, 1.2 Hz, 1″), 6.17 (m, 1 H, 3″), 6.28 (m, 1 H, 2″), 6.32 (t, 1 H, *J* = 6.6 Hz, 1′), 7.68 (d, 1 H, *J* = 1.1 Hz, 6); SIMS (positive ion) *m*/*z* 465 $(M + 1)^+$.

Synthesis of 2,5-Dimethoxy-2,5-dihydrofurfuryl 2′-Deoxy-3′-adenylate (12). A similar procedure for the synthesis of **9** was followed by using 5′-*O*-(dimethoxytrityl)-*N*-benzoyl-2′-deoxyadenosine 3′-(*o*-chlorophenyl) phosphate triethylammonium salt: yield 10%; ^{1}H NMR (D_2O, TSP) δ 2.75 (m, 1 H, 2′), 2.89 (ddd, 1 H, *J* = 14.0, 7.8, 6.1 Hz, 2′), 3.25 (m, 3 H, OCH_3), 3.51 (m, 3 H, OCH_3), 3.84 (dd, 1 H, *J* = 12.8, 3.4 Hz, 5′),

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3.87 (dd, 1 H, J = 12.8, 2.8 Hz, 5'), 3.91 (m, 1 H, 5''), 4.02 (m, 1 H, 5''), 4.37 (ddd, 1 H, J = 3.4, 3.0, 2.8 Hz, 4'), 4.94 (m, 1 H, 3'), 5.66 (m, 1 H, 1''), 6.18 (d, 1 H, J = 6.0 Hz, 3''), 6.26 (m, 1 H, 2''), 6.50 (dd, J = 7.8, 6.5 Hz, 1'), 8.26 (s, 1 H, 2), 8.34 (s, 1 H, 8); SIMS (positive ion) m/z 474 $(M + 1)^+$.

Synthesis of 3'-(3-Pyridazinylmethyl) 2'-Deoxycytidylate (3). A 1-mg sample of 2.5-Dimethoxy-2,5-dihydrofurfuryl 2'-deoxy-3'-cytidylate (**9**) was treated with 1 mL of 0.1 N HCl at room temperature for 1 h, and then the solution was neutralized with 1 N NaOH. To this solution was added 100 μL of 1 N aqueous NH_2NH_2 (pH 7) and the mixture was heated at 90 °C for 5 min.[4a] The resulting solution was concentrated and the residue was purified by HPLC (YMC AQ 302 $5C_{18}$; 0.05 M ammonium formate containing 0–6% acetonitrile, for 20 min; flow rate, 1.5 mL/min; retention time, 8.2 min). **3**: yield 0.53 mg (60%); ^1H NMR (400 MHz, D_2O, TSP) δ 2.27 (ddd, 1 H, J = 14.6, 7.4, 6.7 Hz, 2'), 2.49 (ddd, 1 H, J = 14.6, 6.7, 3.2 Hz, 2'), 3.75 (m, 2 H, 5'), 4.15 (m, 1 H, 4'), 4.75 (m, 1 H, 3'), 5.22 (d, 2 H, J = 8.9 Hz, 3''-CH_2), 6.07 (d, 1 H, J = 7.6 Hz, 5), 6.20 (t, 1 H, J = 6.7 Hz, 1'), 7.84 (d, 1 H, J = 7.6 Hz, 6), 7.87 (dd, 1 H, J = 8.6, 4.9 Hz, 5''), 7.97 (d, 1 H, J = 8.6 Hz, 4''), 9.14 (d, 1 H, J = 4.9 Hz, 6''); SIMS (positive ion) m/z 400 $(M + 1)^+$, 422 $(M + Na)^+$.

Synthesis of 3'-(3-Pyridazinylmethyl) 2'-Deoxyguanylate (4). A similar procedure for the synthesis of **3** was followed by using 2,5-dimethoxy-2,5-dihydrofurfuryl 2'-deoxy-3'-guanylate (**10**): yield 60%; ^1H NMR (400 MHz, D_2O, TSP) δ 2.58 (ddd, 1 H, J = 14.7, 6.4, 2.7 Hz, 2'), 2.77 (m, 1 H, 2'), 3.76 (m, 2 H, 5'), 4.21 (m, 1 H, 4'), 4.94 (m, 1 H, 3'), 5.24 (d, 2 H, J = 6 Hz, 3''-CH_2), 6.23 (t, 1 H, J = 6.4 Hz, 1'), 7.86 (dd, 1 H, J = 9.0, 5.0 Hz, 5''), 7.96 (s, 1 H, 8), 7.98 (d, 1 H, J = 9.0 Hz, 4''), 9.13 (d, 1 H, J = 5.0 Hz, 6''); SIMS (positive ion) m/z 440 $(M + 1)^+$; UV (H_2O) 250 nm (ε 16 200).

Synthesis of 3'-(3-Pyridazinylmethyl) Thymidylate (5). **5** was prepared by a similar procedure from **11**: yield 45%; ^1H NMR (400 MHz, D_2O, TSP) δ 1.89 (s, 3 H, 5-CH_3), 2.35 (m, 1 H, 2'), 2.45 (ddd, 1 H, J = 14.5, 6.5, 3.3 Hz, 2'), 3.73 (dd, 1 H, J = 12.6, 4.8 Hz, 5'), 3.79 (dd, 1 H, J = 12.6, 3.3 Hz, 5'), 4.11 (m, 1 H, 4'), 4.80 (m, 1 H, 3'), 5.23 (d, 2 H, J = 9.0 Hz, 3''-CH_2), 6.23 (t, 1 H, J = 6.5 Hz, 1'), 7.63 (s, 1 H, 6), 7.88 (dd, 1 H, J = 8.0, 4.5 Hz, 5''), 7.98 (d, 1 H, J = 8.0 Hz, 4''), 9.15 (d, 1 H, J = 4.5 Hz, 6''); SIMS (positive ion) m/z 415 $(M + 1)^+$, 437 $(M + Na)^+$.

Synthesis of 3'-(3-Pyridazinylmethyl) 2'-Deoxyadenylate (6). **6** was prepared from **12** by a similar procedure: yield 59%; ^1H NMR (400 MHz, D_2O, TSP) δ 2.64 (ddd, 1 H, J = 14.0, 6.0, 2.4 Hz, 2'), 2.84 (ddd, 1 H, J = 14.0, 8.2, 6.0 Hz, 2'), 3.75 (dd, 1 H, J = 12.7, 3.9 Hz, 5'), 3.81 (dd, 1 H, J = 12.7, 3.1 Hz, 5'), 4.28 (m, 1 H, 4'), 4.95 (m, 1 H, 3'), 5.25 (d, 2 H, J = 8.8 Hz, 3''-CH_2), 6.41 (dd, 1 H, J = 8.3, 6.0 Hz, 1'), 7.86 (dd, 1 H, J = 8.5, 5.0 Hz, 5''), 7.99 (dd, 1 H, J = 8.5, 1.6 Hz, 4''), 8.23 (s, 1 H, 2), 8.30 (s, 1 H, 8), 9.13 (dd, 1 H, J = 5.0, 1.6 Hz, 6''); SIMS (positive ion) m/z 424 $(M + 1)^+$; UV (H_2O) 258 nm (ε 13 000).

Enzymatic Digestion of 7. To a solution of **7** (41 μM, final concentration) in 50 mM Tris-HCl buffer (pH 7.2, 44 μL) were added s.v. PDE (0.15 unit/mL, final concentration) and calf intestine AP (50 unit/mL, final concentration). The solution was incubated at 37 °C for 2 h. HPLC analysis indicated the formation of 2'-deoxycytidine (41 μM, 100%), **4** (35 μM, 85%), and 2'-deoxyguanosine (6 μM, 15%).

Enzymatic Digestion of 8. To a solution of **8** (38 μM) in 50 mM sodium cacodylate buffer (pH 7.0, 50 μL) were added s.v. PDE (0.3 unit/mL) and calf intestine AP (100 unit/mL). The solution was incubated at 37 °C for 2 h. HPLC analysis indicated the formation of 2'-deoxyguanosine (34 μM, 90%), **6** (32 μM, 84%), and **8** (4 μM, 10%).

Stability of 3–6 against Enzymatic Digestion. To a solution containing **3**–**6** (50 μM each) in 50 mM Tris-HCl buffer (pH 7.2) were added s.v. PDE (0.075 unit/mL) and calf intestine AP (50 unit/mL). After a 2-h incubation at 37 °C, the solution was subjected to HPLC analysis in each case. Analysis was carried out on a Cosmosil $5C_{18}$ column (4.6 × 150 mm); elution was with 0.05 M ammonium formate containing a 0–8% acetonitrile linear graident (40 min) at a flow rate of 1.5 mL/min. No digestion of **3** and **5** was observed, whereas compounds **4** and **6** were converted to 2'-deoxyguanosine (3.5%) and 2'-deoxyadenosine (3.9%), respectively, under these conditions.

Detection of 3–6 from Co·PEM-Treated d(CGCGAATTCGCG). The reaction mixture (total volume, 50 μL) contained 1 mM (base concentration) d(CGCGAATTCGCG), 50 mM sodium cacodylate (pH 7.0), and 300 μM green Co·PEM.[8] After irradiation (transilluminator TL 33, 365 nm) for 1 h at 0 °C from a distance of 10 cm, 10 μL of the aliquot was taken up and subjected to HPLC analysis for spontaneously released free bases. Another 10 μL of the aliquot was treated with hydrazine (0.1 M, pH 7.0, 90 °C, 5 min) and the resulting mixture was subjected to enzymatic digestion with s.v. PDE (0.3 unit/mL) and calf intestine AP (100 unit/mL) as described above. The reaction mixture was immediately analyzed by reverse-phase HPLC. Analysis was carried out on a Cosmosil $5C_{18}$ column (4.6 × 150 mm); elution was with 0.05 M ammonium formate containing a 0–8% acetonitrile linear gradient (40 min) at a flow rate of 1.5 mL/min. Quantitation was carried out by comparison with an authentic synthetic sample. The results are listed in Table I.

Detection of 3–6 from Co·PEM-Treated Poly(dA-dT). The reaction mixture (total volume, 50 μL) contained 1 mM (nucleotide concentration) poly(dA-dT), 50 mM sodium cacodylate (pH 7.0), and 300 μM green Co·PEM. After irradiation (transilluminator TL 33) for 1 h at 0 °C from a distance of 10 cm, analysis of spontaneously released free bases was effected by reverse-phase HPLC. Quantitation of pyridazines **3**–**6** was carried out as above. The data are shown in Table I.

Detection of 3–6 from Co·PEM-Treated Calf Thymus DNA. The reaction mixture (total volume, 50 μL) contained 1 mM (nucleotide concentration) calf thymus DNA, 50 mM sodium cacodylate (pH 7.0), and 30 μM green Co·PEM. After irradiation (transilluminator TL 33) for 1 h at 0 °C from a distance of 10 cm, analysis of spontaneously released free bases was effected by reverse-phase HPLC. Quantitation of pyridazines **3**–**6** was carried out as above. HPLC profile on the mixture was shown in Figure 1. The data are shown in Table I.

Detection of 3–6 from Fe(II)·PEM-Treated Calf Thymus DNA. The reaction mixture (total volume, 50 μL) contained 1 mM calf thymus DNA, 50 mM sodium cacodylate (pH 7.0), and 300 μM Fe(II)·PEM. The reaction was initiated by addition of freshly prepared $Fe(NH_4)_2(SO_4)_2$ (3 mM), and the reaction mixture was incubated at 0 °C for 15 min. A 10-μL aliquot was subjected to HPLC analysis for spontaneously released free bases. Another 10-μL aliquot was subjected to hydrazine treatment and subsequent enzymatic digestion as described above. Quantitation of pyridazines **3**–**6** was carried out as above. The data are shown in Table I.

Detection of 4 from NCS-Treated d(CGTACG). The reaction mixture (total volume, 100 μL) contained 1 mM d(CGTACG), 50 mM Tris-HCl (pH 7.2), 4 mM 4-hydroxythiophenol, and 250 μM NCS. In order to dissolve the 4-hydroxythiophenol, methanol was added to the reaction mixture (final concentration of methanol, 0.1%). After incubation at 0 °C for 12 h under aerobic conditions, a 10-μL aliquot was subjected to HPLC analysis for spontaneously released free bases. To another 20-μL aliquot was added 2 μL of 1 N NaOH and the solution was heated at 90 °C for 5 min. The resulting mixture was neutralized and then subjected to HPLC analysis.

Another 20-μL aliquot was treated with hydrazine (0.1 M, pH 7.0, 90 °C, 5 min) and the resulting mixture was subjected to enzymatic digestion with s.v. PDE (0.3 unit/mL) and calf intestine AP (100 units/mL) as described above. The reaction mixture was immediately analyzed by reverse-phase HPLC. Analysis was carried out on a Cosmosil $5C_{18}$ column (4.6 × 150 mm); elution was with 0.05 M ammonium formate containing a 0–8% acetonitrile linear gradient (40 min) at a flow rate of 1.5 mL/min. Quantitation of pyridazine **4** was carried out as above. The results are shown in Table II.

Detection of 3–6 from NCS-Treated Calf Thymus DNA. The reaction mixture (total volume, 100 μL) contained 1 mM calf thymus DNA, 50 mM Tris-HCl (pH 7.2), 4 mM 4-hydroxythiophenol, and 250 μM NCS. After incubation at 0 °C for 12 h, a 10-μL aliquot was subjected to HPLC analysis for spontaneously released free bases. To another 20-μL aliquot was added 2 μL of 1 N NaOH and the solution was heated at 90 °C for 5 min. The resulting mixture was neutralized and then subjected to HPLC analysis. Another 20-μL aliquot was subjected to hydrazine treatment and subsequent enzymatic digestion as described above. The aliquot was treated with hydrazine (0.1 M, pH 7.0, 90 °C, 5 min) and the resulting mixture was subjected to enzymatic digestion with s.v. PDE (0.3 unit/mL) and calf intestine AP (100 units/mL) as described. The reaction mixture was immediately analyzed by reverse-phase HPLC. Quantitation of pyridazines **3**–**6** was carried out as described above. The results are shown in Figure 3 and Table II.

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