Chemistry of Neocarzinostatin-Mediated Cleavage of Oligonucleotides. Competitive Ribose C5′ and C4′ Hydroxylation

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Abstract: A series of hexanucleotides possessing A-T, G-C, inosine (I)-C, and 2-aminoadenine (A^{NH_2})-T base pairs at the 5′-side of the target thymine were prepared, and their selectivity for C-5′ and C4′ oxidation in the neocarzinostatin (NCS)-mediated degradation was investigated. Quantitative product analysis indicated that preferential C5′ oxidation of the deoxyribose moiety of the target T occurs at -5′-AT- and 5′-IT- sites, whereas C5′ and C4′ oxidation occurs competitively at T of -5′-GT- and -5′-A^{NH_2}T- sites. On the basis of the cleavage data, a binding model that permits competitive hydrogen abstraction from C5′ and C4′ of the deoxyribose moiety has been proposed. Computer modeling of the hexamer duplex and the post-activated form of the NCS chromophore (**3**) suggested that, in the complex between $d(GCATGC)_2$ and **3**, the C6 carbon of **3** is close to the target C5′ (*pro-S*) hydrogen. In contrast, the tricyclic core of **3** is slightly lifted up so as to become closer to the C4′ hydrogen in the complex between duplex d(GCGTGC)/d(CGCACG) and **3** due to the van der Waals contact between the protruding guanine 2-amino group in the minor groove and the core moiety of **3**.

Neocarzinostatin (NCS) is an antitumor antibiotic consisting of nonprotein chromophore (NCS-C) and its carrier protein.[1] NCS-C undergoes irreversible reaction with thiols to generate a biradical species which is capable of cleaving DNA via hydrogen abstraction from the DNA sugar backbone with a high degree of base specificity (T > A ≫ C ~ G) upon aerobic incubation.[1b,c,2] NCS-C consists of three main structural subunits, a substituted naphthoate group, an amino sugar (*N*-methyl-α-D-fucosamine) and a highly strained bicyclo[7.3.0]dodecadiendiyne epoxide unit (Figure 1).[3] NCS-C binds to double stranded DNA via intercalation of its naphthoate moiety and interaction of the diendiyne bicyclic core with the minor groove.[1b,c,4] A diradical species **2** derived from the diendiyne epoxide moiety via nucleophilic addition of a thiol at C12 has been proposed to abstract hydrogen from the deoxyribose moiety of DNA to induce DNA strand cleavage in the presence of oxygen (Scheme I).[1b,5] The prominent DNA damage is direct strand breaks at -5′-AT- sites resulting in the formation of thymidine 5′-aldehyde fragments at the 5′-termini and phosphate at the 3′-termini.[6] It has also been shown that oxidation at C1′ of the deoxyribose-giving 2- deoxyribonolactone abasic site also occurs at the -5′-AGC- sequence as a less prevalent lesion.[7] Recently, partial incorporation of deuterium from C5′-deuterium-labeled oligonucleotide into the C6 position of **3** has been demonstrated.[8]

We have very recently found that there are two distinct cycloaromatization pathways (paths A and B) in the activation of NCS-C by thiol under physiological conditions as outlined in Scheme I.[9] Incubation of NCS-C with 2-mercaptoethanol in the presence of apoprotein in aqueous buffer solution produced a previously unobserved cyclization product **4** as a major product via ionic pathway (path B).[9] We have also demonstrated that the use of hexanucleotides as a sequence-specific substrate for NCS provides a very useful tool for understanding the detailed chemistry of NCS-mediated DNA degradation.[10] By using a self-complementary hexanucleotide $d(GCATGC)_2$, we were able to characterize the structure of the previously unidentified oxidized deoxyribose moiety associated with spontaneous free base release.[10a] We also observed that previously unobserved C4′ hydroxylation of the deoxyribose moiety does occur significantly at T_3 of a self-complementary hexanucleotide $d(C_1G_2T_3A_4C_5G_6)_2$ in competition with C5′ oxidation at A_4 (Scheme II).[10b] This type of C4′ hydroxylation has also been observed in NCS-mediated degradation of calf thymus DNA.[11] Specific detection methods for C4′ hydroxylated abasic sites recently developed in our laboratory have indicated that C4′ hydroxylation is estimated to be a minimum of 17% of the total event that occurred by the action of NCS on calf thymus DNA.[11] More recently Goldberg and co-workers have indicated that NCS-C abstracts the C4′ hydrogen of the T residue at a d(GT) step in d(TCTTTGA), d-(TTCTCATGTTTGA), and the HindIII-BamH1 restriction fragment (322 bp) of pBR322.[12]

In order to get insight into the binding geometry that permits competitive hydroxylation at C5′ and C4′ of the deoxyribose moiety, a series of hexanucleotides possessing A-T, G-C, inosine (I)-C, and 2-aminoadenine (A^{NH_2})-T base pairs at the 5′-side of the target thymine were prepared and their selectivity for C5′ and C4′ oxidation in the NCS-mediated degradation was investigated (Figure 2). Careful analysis of NCS-mediated degradations of

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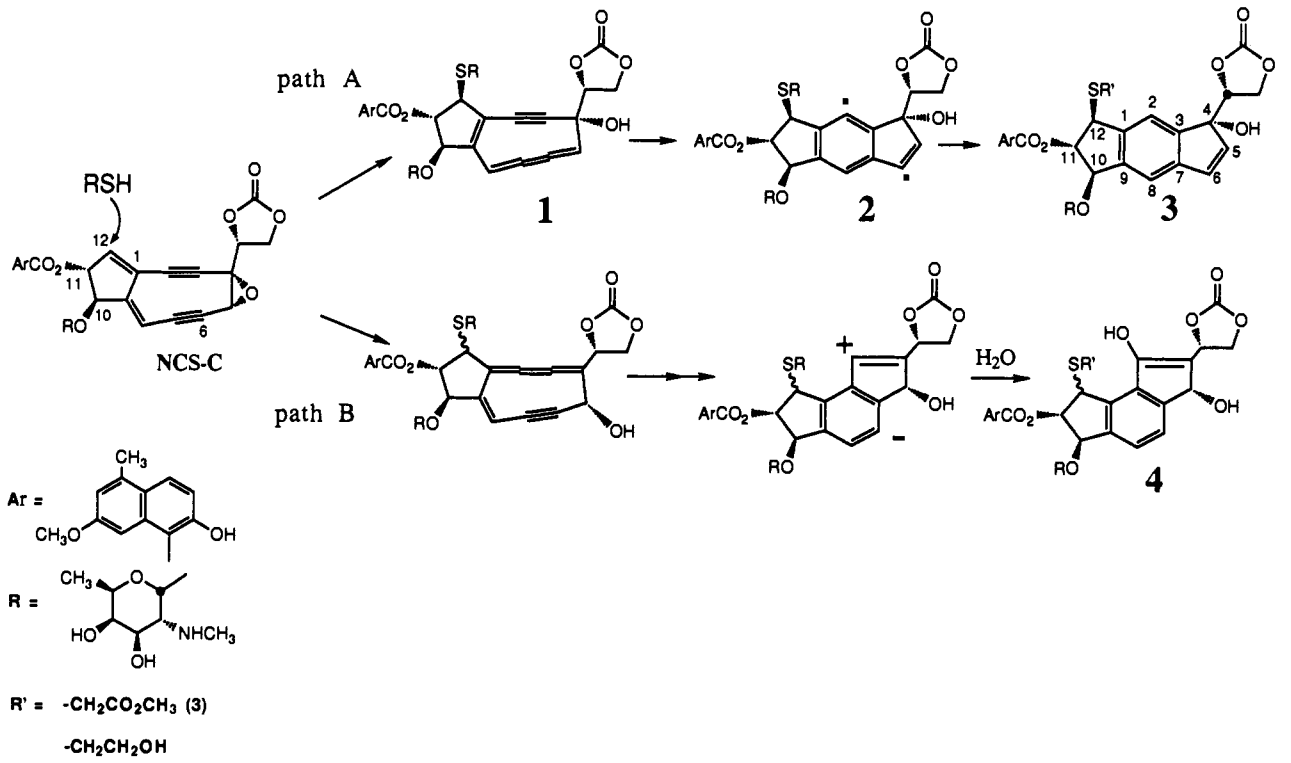
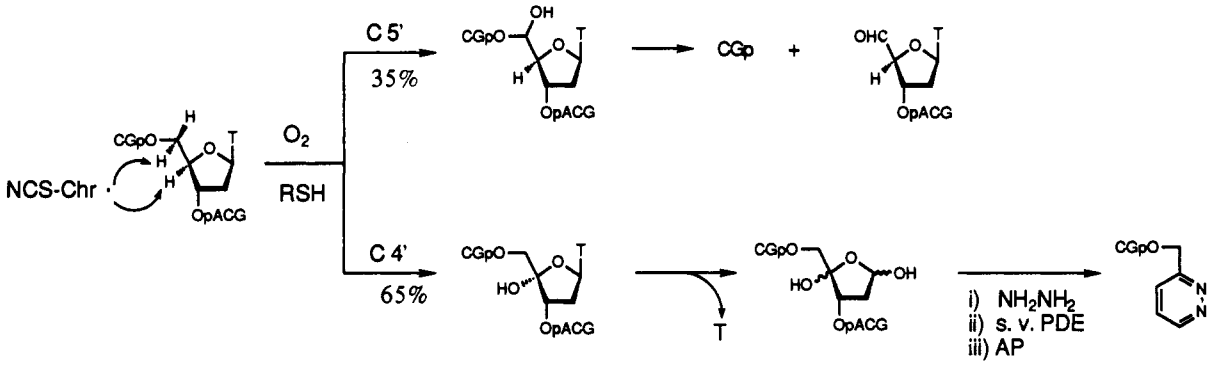
Scheme I



Scheme II



these oligomers indicated that the ratio of the selectivity for C5′ vs C4′ oxidation is strongly dependent on the depth of the minor groove at the 5′-side of the target thymine. Described herein are detailed experimental results, in conjunction with computer-modeling studies, which may provide important insight into the NCS–DNA association and explain the base specificity for the DNA cleavage.

In a typical experiment, a mixture containing a self-complementary hexanucleotide $d(G_1C_2A_3T_4G_5C_6)_2$, NCS, and 4-hydroxythiophenol (HTP)[10] as an activator in Tris-HCl buffer (pH 7.2) was incubated at 0 °C for 12 h under aerobic conditions. Direct HPLC analysis of the reaction mixture indicated a clean formation of d(GCA)p and thymidine 5′-aldehyde fragment d(T*GC) together with minor amounts of spontaneously released thymine and adenine, showing that NCS recognizes the -5′-AT- site and the thiol-activated NCS-C diradical abstracts hydrogen from the deoxyribose moiety specifically at T_4 of this hexamer.[10a]

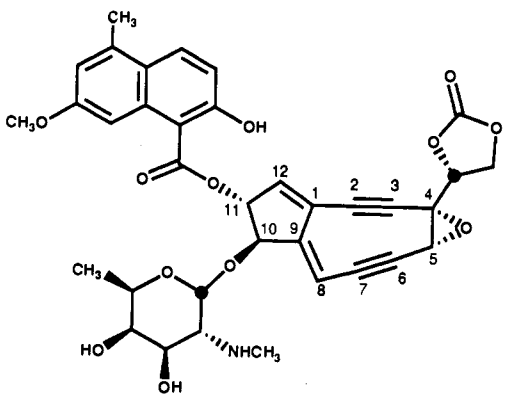


Figure 1. Structure of the neocarzinostatin chromophore (NCS-C).

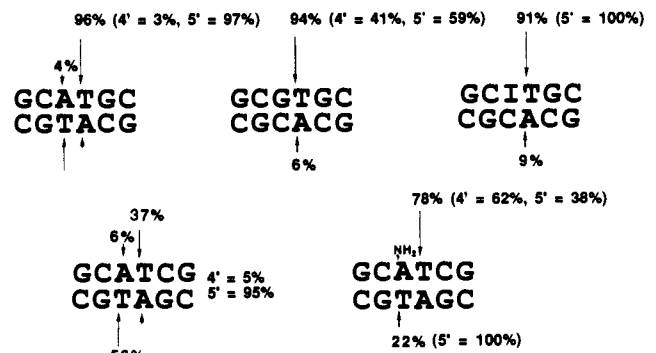


Figure 2. Cleavage sites of NCS-mediated degradation of hexanucleotides. The arrows represent the location and extent of cleavage. Selectivity for C4′ vs C5′ oxidation is shown in parentheses.

Scheme III

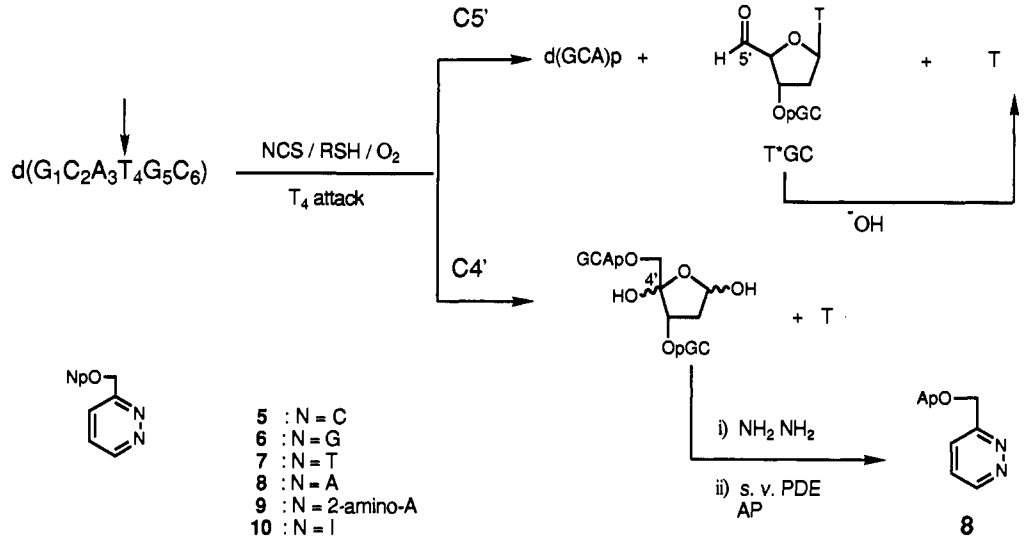


Table I. Quantitative Analysis of Products Formed in Neocarzinostatin-Mediated Degradation of Hexanucleotides

run no.	substrate	free base,[a] μM			pyridazine,[b] μM							ratio (C5′ vs C4′)
		T	A	total	5(C)	6(G)	7(T)	8(A)	9(A^{NH_2})	10(I)	total	
1	$d(GCATGC)_2$	2.3 (30.4)	1.2 (1.3)	3.5 (31.7)	0	0	0	0.8			0.8	97:3
2	d(GCGTGC) d(CGCACG)	8.0 (23.2)	0 (1.6)	8.0 (24.8)	0	9.4	0.3	0.2			9.9	59:41
3	d(GCITGC) d(CGCACG)	4.3 (17.9)	0 (0)	4.3 (17.9)	0.4	0	0	0.9		0	1.3	100:0
4	d(GCATCG) d(CGTAGC)	1.4 (26.2)	0 (2.0)	1.4 (28.2)	0	0	0	1.3			1.3	95:5
5	$d(GCA^{NH_2}TCG)$	11.0 (25.3)	0 (0)[c]	11.0 (25.3)	0	0	0		12.2		12.2	38:62
	d(CGTAGC)		0 (0)					0			0	100:0

[a] Spontaneously released bases were determined by HPLC. The values in parentheses are the amount of free bases after alkali treatment (0.5 N NaOH, 90 °C). [b] Quantitated as corresponding nucleosides after treatment with snake venom phosphodiesterase and AP by reverse phase HPLC. [c] Formation of 2-aminoadenine.

When the reaction mixture was treated with hot alkali (90 °C, 5 min), quantitative release of thymine from d(T*GC) was observed. Therefore, the total event that occurred at T_4 was easily determined by quantitation of the total amount of released thymine after hot alkali treatment. The amount of the deoxyribose C4′ oxidation at T_4 was determined by quantitation of A-pyridazine **8** after hydrazine treatment and subsequent enzymatic digestion as described previously.[10b,11] Under this condition, the 3′-phosphoglycolate termini such as GCA-glycolate was not detected, suggesting that the C4′-hydroperoxide species formed by trapping of the C4′-radical by O_2 is efficiently reduced by HTP to afford the C4′-hydroxy species before hydroperoxide can fragment to phosphoglycolate. These results also indicate that the extent of C4′ oxidation at T_4 is correctly estimated by the amount of **8**. No phosphoglycolate termini were detected in the cleavage of all duplex hexamers tested in this study utilizing HTP as an activator.[12b] Therefore, in all cases C4′ oxidation was assayed by the amount of pyridazine produced. It was also reported that no significant amount of phosphoglycolate termini is produced at T_9 in the cleavage of d(TTCTCATGTTTGA) when HTP was used as an activator.[12a] The portion of C5′ oxidation at T_4 was obtained from the total amount of released thymine after hot alkali treatment (total event occurring at T_4) minus the amount of **8** (C4′ oxidation), which corresponds to the amount of d(GCA)p produced (Scheme III). The amount of **8** was always less than that of spontaneously released thymine. This may be due to the Criegee type decomposition of C5′ hydroperoxide intermediate which was reported previously.[10a] The ratio of C5′ vs C4′ oxidation at T_4 of d(GCATGC) thus obtained was 97:3 (Table I, run 1). For the analysis of the cleavage of modified base-containing oligomers such as d(CGITCG)/d(CGCACG) and d-($GCA^{NH_2}TCG$)/d(CGTAGC), 2-amino-3′-(3-pyridazinylmethyl)-2′-deoxyadenylate (**9**) and 3-pyridazinylmethyl 2′-deoxyinosine-3′-monophosphate (**10**) were prepared independently. Under the standard enzymatic digestion conditions for the assay of C4′ hydroxylated abasic site, both **9** and **10** were stable. The selectivity ratios for C5′ vs C4′ oxidation of various hexanucleotides were examined as described above. The results are summarized in Figure 2 and Table I.

When a heteroduplex $d(G_1C_2G_3T_4G_5C_6)/d(C_{12}G_{11}C_{10}A_9C_8G_7)$ was incubated with NCS under the same conditions, the d-(GCGTGC) strand was selectively oxidized with a remarkable increase of spontaneously released thymine (run 2). In fact, a comparable amount of G-pyridazine **6**[10b,11] to that of the released thymine was detected. The selectivity ratio for C5′ vs C4′ oxidation at T_4 was 59:41 (run 2). In a marked contrast, when $d(G_1C_2I_3T_4G_5C_6)/d(C_{12}G_{11}C_{10}A_9C_8G_7)$ possessing the I-C base pair instead of the G-C pair at the 5′-side of the target T_4 was used as a substrate, cleavage at T_4 occurred exclusively via the C5′ pathway (run 3). These results indicate that the presence of guanine 2-amino group of G_3 of d(GCGTGC)/d(CGCACG) dramatically increases the ratio of C4′ to C5′ oxidation. Since the guanine 2-amino group of the G-C base pair was protruded into the minor groove thus shallowing the minor groove, the binding of activated NCS-C to the minor groove of d-(GCGTGC)/d(CGCACG) would be sterically more hindered and the binding orientation would be slightly different from that for the binding to d(GCITCG)/d(CGCAGC) or $d(GCATGC)_2$ which does not possess the 2-amino group in the minor groove of the 5′-side of the cleavage site (Figure 3).

A more distinct example for the dramatic effect of the 2-amino group in the minor groove on the ratio for C5′ vs C4′ oxidation was the cleavage of a heteroduplex $d(G_1C_2A_3{}^{NH_2}T_4C_5G_6)/d-(C_{12}G_{11}T_{10}A_9G_8C_7)$ (run 5) in comparison with the cleavage of d(GCATCG)/d(CGTAGC) (run 4). The former duplex has the 2-aminoadenine (A^{NH_2})-T base pair instead of the A-T base pair of the latter. Thermodynamic experiments of the oligomers indicated that $d(GCA^{NH_2}TCG)$/d(CGTAGC) forms a more stable

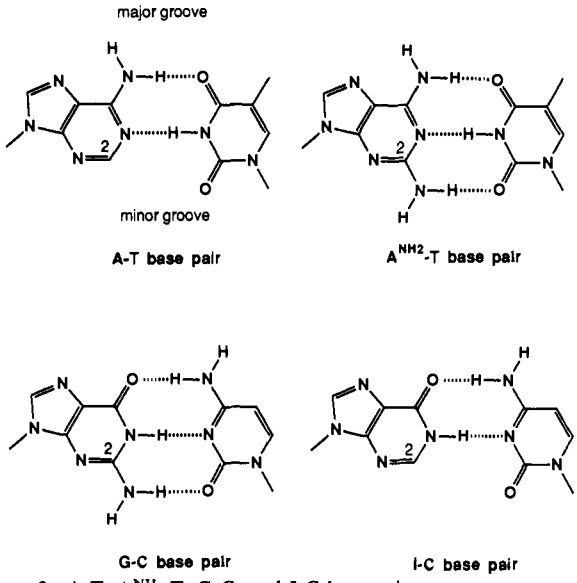


Figure 3. A-T, A^{NH_2}-T, G-C, and I-C base pairs.

duplex (T_m 42.8 °C) than the parent duplex d(GCATCG)/d(CGTAGC) (T_m 31,4 °C), suggesting that the 2-amino group of A^{NH_2} is participating in an extra hydrogen bonding with complementary T_{10}. A recent ^1H NMR study of a 2-aminoadenine-containing decamer showed that the 2-amino group does not disturb the global or local conformation of the DNA duplex.[13] The amount of spontaneously released thymine from the former duplex was increased significantly compared to that from d(GCATCG)/d(CGTAGC), resulting in the formation of a considerable amount of 2-aminoadenine-pyridazine **9**. The selectivity ratio for C5′ vs C4′ oxidation for the cleavage at T_4 of the d($G_1C_2A_3{}^{NH_2}T_4C_5G_6$) strand was 38:62, whereas the cleavage of the d($C_{12}G_{11}T_{10}A_9G_8C_7$) strand at T_{10} was exclusively via the C5′ pathway. These results indicate that when the minor groove at the 5′-side of the target thymine is relatively deep such as that for the -5′-AT- or 5′-IT- sequence, the oxidation at the target thymine occurs exclusively via the C5′ pathway, whereas if the minor groove becomes shallow due to the protruded 2-amino group as seen in a -5′-GT- or -5′-A^{NH_2}T- step, competitive oxidation occurs at C5′ and C4′ of the deoxyribose moiety of the target thymine. The present results are entirely consistent with the previous observations, i.e., C4′ oxidation was not observed in the cleavage of poly(dAdT) and C4′ oxidation occurred preferentially at -5′-GT- and -5′-CT- sites in the cleavage of calf thymus DNA.[10b,11] A recent study by Goldberg and co-workers has demonstrated that the substitution of deuterium for hydrogen at C4′ or C5′ of deoxyribose causes a considerable shift of the attacking rate at each position.[12] Also the total cleavage product per cleavage site stayed almost constant (Table I). These results clearly suggest that the distance between the radical center generated on the NCS-C core and the deoxyribose C4′ and C5′ hydrogens is strongly dependent on whether the *N*-2 amino group is present or not in the minor groove at the 5′-side of the target thymine (Figure 3).

These experimental results led us to propose a binding model for the activated NCS-C–DNA complexes based on a computer-assisted molecular modeling. The naphthoate group of NCS-C has been known to intercalate into base pairs at the AT-rich region of DNA.[1,4,14] The unwinding angle of 21° and the vertical lengthening of 3.3 Å have been estimated.[14a] It has already been shown that NCS activation by thiol occurs via nucleophilic addition to C12 to result in the formation of an ene–yne–cumulene system

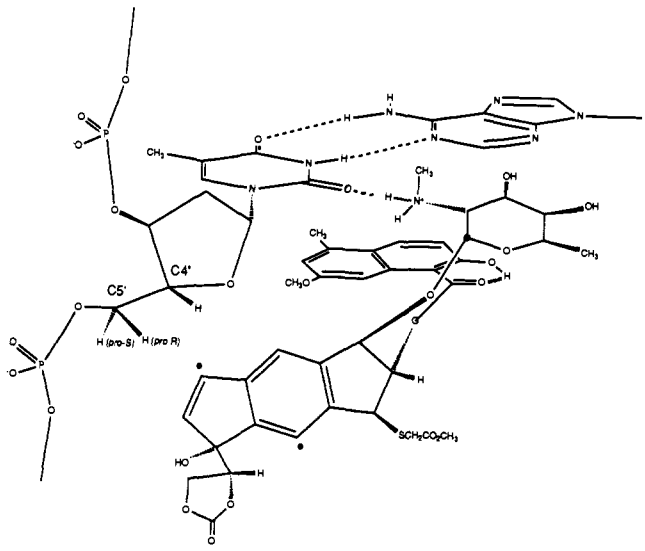


Figure 4. Schematic drawing of the proposed mechanism for the specific recognition of the thymine residue by the post-activated form of NCS-C (**3**). Hydrogen bonding is represented by dashed lines.

Table II. Calculated Distances between the C6 Carbon of **3** and the Deoxyribose Hydrogen of Thymine$_4$ (T_4)[a]

oligomer	deoxyribose hydrogen		
	C4′	C5′(*pro-R*)	C5′(*pro-S*)
d(GCATGC)$_2$	4.47	3.12	4.68
d(GCGTGC) d(CGCACG)	3.27	3.13	4.49

[a] Distances in Å calculated by energy minimization by AMBER.[15]

1 which may spontaneously rearrange to a diradical species **2** possessing radical centers at C2 and C6.[5b] Hydrogen abstraction by these radicals from the deoxyribose has been proposed to induce DNA strand cleavage.[5,7d] In fact, the labeled C5′ hydrogen of the deoxyribose residue recently has been shown to be incorporated into the C6 position of **2**.[8] Since the absolute stereochemistry (C10(*R*)–C11(*R*)) and the conformation of **3** in solution have been defined by Myers et al. by means of NOE,[3c] we consider the complexes of the post-activated form of NCS-C (**3**) with two B-form duplexes, d(GCATGC)$_2$ and d(GCGTGC)/d(CGCACG). Only the binding to the minor groove was considered, since both C4′ and C5′ deoxyribose hydrogens are not accessible in the major groove. Actually, there is indirect evidence for the minor groove binding.[14]

Given the experimental data for the selectivity for C4′ vs C5′ oxidation of these two duplexes, the aim of the model building was to find the stable intercalation site for **3** in the minor groove of d(GCGTGC)/d(CGCACG) for which either the C2 or the C6 radical of **2** is close enough to both C5′ and C4′ hydrogens of T_4 to permit competitive hydrogen abstraction. In the case of binding to the minor groove of d(GCATGC)$_2$, one of the radical centers of **2** must be closer to the C5′ hydrogen than to the C4′ hydrogen of T_4. Computer-modeling studies using AMBER[15] suggest that when the naphthoate group intercalates between the A3-T4′/T4-A3′ base pair of d($G_1C_2A_3T_4G_5C_6$)/d($C_6{}'G_5{}'T_4{}'A_3{}'C_2{}'G_1{}'$), the methylamino group of the sugar moiety, which is presumably protonated under neutral pH,[4] lies in the minor groove so as to form a hydrogen bonding with the 2-carbonyl oxygen of the thymine base at T_4 (Figure 4). This interaction would guide the tricyclic core of **3** sliding into the minor groove of the 5′-side of T_4. Consequently, the C6 carbon of **3** is closer to the C5′ (*pro-S*) hydrogen. The energy-minimized complex between d(GCATGC)$_2$ and **3** using AMBER was shown in Figure 5a. The calculated distance between the C6 carbon and the C5′ (*pro-R*) hydrogen of T_4 is 3.12 Å, whereas the distance between C6 and C4′ hy-

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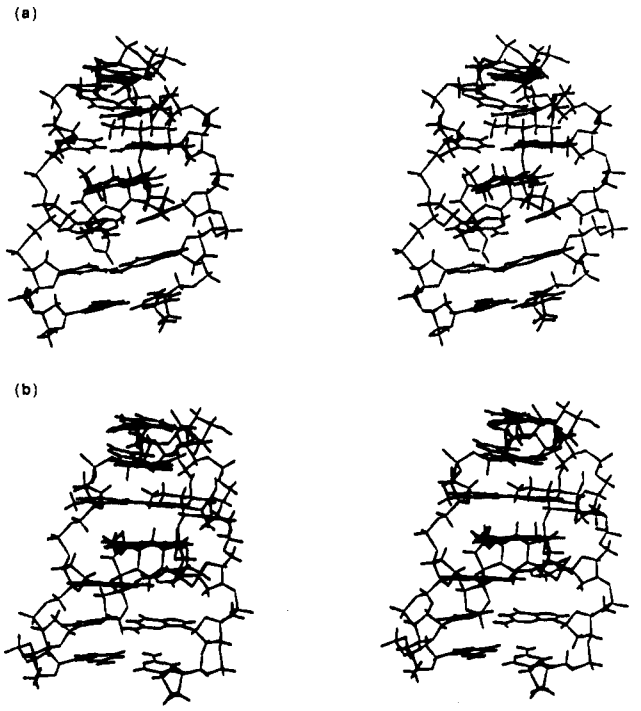

Figure 5. Computer-generated depiction of the proposed association of the post-activated form of NCS-C (3) with the minor groove of hexanucleotides which are energy minimized by AMBER.[15] (a) Stereodrawing of 3 and d(GCATGC)$_2$. (b) Stereodrawing of 3 and d(GCGTGC)-d(CGCACG), wherein the tricyclic core of 3 in the minor groove is slightly lifted up so as to become closer to the C4′ hydrogen.

drogens is longer (4.47 Å) (Table II). If the AT base pair at the C5′-side of the target thymine is replaced by the GC base pair, i.e., 3 forms a stable complex with d(GCGTGC)/d(CGCACG) in a similar manner as with d(GCATGC)$_2$, then the tricyclic core is slightly lifted up so as to become much closer to the C4′ hydrogen (3.27 Å) due to van der Waals contact between the guanine 2-amino group in the minor groove and the bulky core moiety (Figure 5b). This mode of association is consistent with the observed high ratio of C4′ oxidation at T_4 of this heteroduplex.

The present modeling studies provide interesting predictions: (1) it is the C6 radical of putative diradical 2 that is responsible for hydrogen abstraction from C5′ and C4′ positions of the deoxyribose moiety. Hydrogen abstraction from C5′ by the C6 radical of 2 was actually proved by recent labeling experiments.[8] (2) The methylamino group of the sugar moiety of NCS-C is participating in the specific recognition of thymine residue by forming a hydrogen bond with the C-2 carbonyl of thymine base at the target site (Figure 4). So far, there has been no hypothesis for the mechanism of specific recognition of thymine residue by NCS-C, although the amino sugar moiety has been proposed to interact with the DNA phosphate backbone.[4] Previous modeling studies indicated a similar complex between 3 and d(GAGCG)/d(CGCTC) which may allow for simultaneous attack of the diradical 2 at the C1′ of cytosine of one strand and the C5′ of thymine of the other strand.[8,16] However, there was no explanation for the specific recognition of thymine residue by NCS-C. While other intercalation models which can give rise to hydrogen abstraction from the C5′ of deoxyribose at the 5′ site of the target thymine as a single lesion are plausible,[16] the selectivity ratio for C5′ vs C4′ oxidation shown in Figure 2 is best explained by the model depicted in Figures 4 and 5.

In conclusion, we have demonstrated that competitive oxidation at C5′ and C4′ of the deoxyribose moiety occurs efficiently only when the purine 2-amino group is present in the minor groove at the 5′-side of the reacting thymine as observed with d(GCGTGC)/d(CGCACG) and d(GCA^{NH_2}TCG)/d(CGTAGC). On the basis of the experimental results, an intercalation model that permits competitive hydrogen abstraction from C5′ and C4′ of the deoxyribose has been proposed. The energy-minimized complex between d(GCATGC)$_2$ and the post-activated form of NCS-C (3) by using AMBER has indicated that the methylamino group of the sugar moiety of NCS-C is participating in the specific recognition of the thymine residue by hydrogen bonding.

Experimental Section

Materials and Methods. Neocarzinostatin was obtained from the Pola Kasei Co. Ltd. The concentration of NCS was determined spectrophotometrically (ϵ_{340} 10800 mM^{-1} cm^{-1}).[14a] Calf intestine alkaline phosphatase (AP, 1000 unit/mL) and snake venom phosphodiesterase (s.v. PDE, 3 unit/mL) were purchased from Boehringer Mannheim. Silica gel column chromatography was carried out on Wakogel C-200. Preparative TLC was carried out on Merck silica gel 60 PF$_{254}$ 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. 2-Amino-*N*-2,*N*-6-dibenzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxyadenosine was prepared from 2-aminoadenosine by benzoylation[17] followed by conventional deoxygenation[18] and dimethoxytritylation.[17] 2-Cyanoethylphosphoramidite of 2-amino-*N*-2,*N*-6-dibenzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxyadenosine was prepared by the procedure of van Boom[19] and directly applied to automated DNA synthesizer without further purification.

Synthesis of Deoxyhexanucleotides. Oligonucleotides were prepared by the β-(cyanoethyl)phosphoramidite method[20] on controlled pore glass supports (1 μmol) by using an ABI 381 A DNA synthesizer. After automated synthesis, the oligomer was detached from the support by soaking in concentrated NH_4OH for 1 h at room temperature. Deprotection was normally carried out by heating the concentrated aqueous NH_4OH solution for 12 h at 55 °C. In the case of A^{NH_2}-containing oligomer, more drastic conditions for deprotection (24 h, 65 °C) were necessary to remove the *N*-2-benzoyl group. Aqueous NH_4OH was then removed by evaporation, and the crude oligomer was purified by reverse phase HPLC (Cosmosil 5C$_{18}$ column; 0.05 M triethylammonium acetate containing 3–15% acetonitrile/20 min linear gradient; flow rate, 1.5 mL/min). After lyophilization, approximately 20 OD of pure oligomer was isolated. Purity and concentration of all oligonucleotides were determined by complete digestion with s.v. PDE and AP to 2′-deoxymononucleosides. The melting temperature (T_m) of d(GCA^{NH_2}TCG)-d(CGTAGC) was 42.8 °C at 0.198 mM base concentration, whereas T_m of d(GCATCG)-d(CGTAGC) was 31.4 °C at 0.198 mM base concentration.

Synthesis of Pyridazine Mononucleotides 5–10. Pyridazine mononucleotides 5–8 were prepared according to the previous method.[11]

(a) Synthesis of 2-Amino-3′-(3-pyridazinylmethyl)-2′-deoxyadenylate (9). A mixture of 2-cyanoethylphosphoramidite of 2-amino-*N*-2,*N*-6-dibenzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxyadenosine (39 mg, 0.04 mmol) and 2,5-dimethoxy-2,5-dihydrofurfuryl alcohol (15 mg, 0.1 mmol) was dried by coevaporation with acetonitrile (three times) and redissolved in 400 μL of acetonitrile. To this solution was added 100 μL of 0.5 M tetrazole in acetonitrile, and the mixture was stirred for 30 min at room temperature under argon. The coupling product was then oxidized by adding 1 mL of I_2 solution (I_2/H_2O/pyridine/tetrahydrofuran, 3:2:9:76), and the solution was kept standing for 30 min at room temperature. After evaporation of the solvent, the residue was extracted with dichloromethane. The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The crude product thus obtained was treated with 80% aqueous acetic acid (50 mL) at room temperature for 1 h, and the solvent was evaporated under reduced pressure. The residue was treated with concentrated aqueous ammonia–pyridine (2:1, 10 mL) at 65 °C for 24 h and then concentrated. The resulting residue was subjected to preparative TLC (silica gel, 2-propanol–concentrated aqueous ammonia, 3:2). The crude product was dissolved in 1 mL of 0.1 N HCl and kept standing for 1 h at room temperature. To this solution was added 100 μL of 1 N aqueous NH_2NH_2 (pH 7.0), and the solution was kept standing for 5 h at room temperature. The resulting solution was concentrated and the residue was purified by HPLC (Cosmosil 5C$_{18}$ column; 0.05 M ammonium formate containing 0–8% acetonitrile/40 min linear gradient;

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flow rate, 1.5 mL/min; retention time, 32.8 min). After lyophilization, 2-amino-3′-(3-pyridazinylmethyl)-2′-deoxyadenylate (**9**) was obtained as a white powder; yield, 1 mg (4.2% from phosphoramidite). The purity of **9** was more than 95% as determined by HPLC analysis. ^{1}H NMR (D_2O, TSP) δ 2.57 (ddd, 1 H, J = 14.2, 5.9, 1.8 Hz, 2′), 2.78 (ddd, 1 H, J = 14.2, 8.0, 6.1 Hz, 2′), 3.74 (dd, 1 H, J = 12.6, 3.9 Hz, 5′), 3.79 (dd, 1 H, J = 12.6, 3.1 Hz, 5′), 4.24 (m, 1 H, 4′), 4.90 (m, 1 H, 3′), 5.25 (d, 2 H, J = 8.8 Hz, 3″CH_2), 6.25 (dd, 1 H, J = 8.0, 5.9 Hz, 1′), 7.86 (dd, 1 H, J = 8.6, 5.0 Hz, 5″), 7.98 (s, 1 H, 8), 7.99 (m, 1 H, 4″), 9.12 (dd, 1 H, J = 5.0, 1.3 Hz, 6″); FABMS (positive ion) m/z 439 $(M + 1)^+$, 461 $(M + Na)^+$, (negative ion) m/z 437 $(M - 1)^-$.

(b) Synthesis of 3-Pyridazinylmethyl 2′-Deoxyinosine-3′-monophosphate (10). A similar procedure for the synthesis of **9** was followed by using 2-cyanoethylphosphoramidite of 5′-*O*-dimethoxytrityl-2′-deoxyinosine; yield, 8.8% from phosphoramidite. The purity of **10** was more than 95% as determined by HPLC analysis. ^{1}H NMR (D_2O, TSP) δ 2.69 (ddd, 1 H, J = 14.2, 6.0, 2.8 Hz, 2′), 2.84 (ddd, 1 H, J = 14.2, 7.8, 6.6 Hz, 2′), 3.76 (dd, 1 H, J = 12.9, 3.5 Hz, 5′), 3.79 (dd, 1 H, J = 12.9, 4.5 Hz, 5′), 4.28 (m, 1 H, 4′), 4.96 (m, 1 H, 3′), 5.26 (d, 2 H, J = 8.8 Hz, 3″CH_2), 6.42 (dd, 1 H, J = 7.8, 6.0 Hz, 1′), 7.88 (dd, 1 H, J = 8.6, 5.0 Hz, 5″), 7.99 (dd, 1 H, J = 8.6, 1.7 Hz, 4″), 8.21 (s, 1 H, 2), 8.30 (m, 1 H, 4″), 9.14 (dd, 1 H, J = 5.0, 1.7 Hz, 6″); FABMS (positive ion) m/z 425 $(M + 1)^+$, (negative ion) m/z 423 $(M - 1)^-$.

Stability of 9 and 10 against Enzymatic Digestion. To a solution containing **9** and **10** (50 μM each) in 50 mM Tris-HCl buffer (pH 7.2) were added s.v. PDE (0.3 unit/mL) and calf intestine AP (100 unit/mL). After 2 h of 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) with a linear gradient (40 min) at a flow rate of 1.5 mL/min. Compounds **9** and **10** were converted to 2-amino-2′-deoxyadenosine (5.6%) and 2′-deoxyinosine (6.5%), respectively, under the above conditions.

Quantitative Product Analysis of NCS-Treated Deoxyhexanucleotides. Each of the reaction mixtures (total volume 75 μL) containing deoxyhexanucleotide (1.33 mM, base concentration) and NCS (333 μM) in 66.7 mM Tris-HCl (pH 7.2) were prepared. To each reaction mixture was added 25 μL of 4-hydroxythiophenol solution (16 mM in H_2O containing 0.4% methanol) was added to initiate the reaction. After incubation at 0 °C for 12 h under aerobic conditions, 10 μL of the aliquot was subjected to HPLC analysis for spontaneously released free bases. To another 20 μL of the 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 of the aliquot was subjected to hydrazine treatment and subsequent enzymatic digestion. 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 before.[11] The reaction mixture was immediately analyzed by reverse-phase HPLC. Analysis was carried out on a Cosmosil $5C_{18}$ column. Elution was with 0.05 M ammonium formate containing 0–8% acetonitrile, linear gradient (40 min) at a flow rate of 1.5 mL/min. The results are shown in Table I.

The extent of cleavage at individual sites was determined by the amount of released free base after alkaline treatment (0.1 N NaOH, 90 °C, 5 min) divided by the amount of the total event that occurred. Under this condition C5′ cleavage products such as 5′-aldehyde fragments were quantitatively converted to the corresponding free bases. Thus, the total event induced by NCS was equal to the sum of all free bases. In the cases of d(GCATCG)-d(CGTAGC) (run 4) and d(GCA^{NH_2}TCG)-d(CGTAGC) (run 5), a proportion of the cleavage within the two thymine residues in the duplex (cleavage at T_4 and T_{10}) was estimated from the amount of the cleavage fragments after alkaline treatment. For example, the cleavage ratio for T_4 vs T_{10} was determined as 40:60 by the amount of d(GCA)p and d(CGA)p. The C5′ vs C4′ cleavage ratio was obtained from the amounts of the pyridazine and the cleavage products.

Molecular Modeling Studies. All calculations were performed with the AMBER program[15] by using all-atom force-field parameters.[21] A starting structure of **3** was constructed by assorting the appropriate partial structures. The coordinates of 1,3-dioxolan-2-one, naphthalene, tricyclic ring, and sugar moieties were retrieved from the Cambridge Structural Database.[22] The connecting parts between them were built using standard bond lengths and angles. The molecule has rotational freedom around the connecting bonds. The most plausible conformations were selected by inspection of non-bonded contacts. The conformational regularity observed in carboxylic esters derived from secondary alcohols indicates that the parallel orientation of naphthoate ester carbonyl and the methine C_{11}-H is preferable.[23] This conformation was adopted in the starting structure. The assembled initial structure was energy minimized using a distance-dependent dielectric constant of $\epsilon = r_{ij}$ (r_{ij} stands for the distance between atoms i and j) and the convergence criteria with the root-mean-square gradient of less than 0.1 kcal/(mol Å). The cutoff distance for nonbonded pairs was set to be 5 Å. The starting coordinates of duplex hexanucleotides, $d(GCATGC)_2$ and d(GCGTGC)/d(CGCACG), were built on the basis of the X-ray coordinates of the complex between netropsin and $d(CGCGAATTCGCG)_2$[24] by substituting relevant bases. The minimized structure of **3** was docked visually to these duplexes near T_4 with the use of the interactive graphics program MOGLI[25] to search for reasonable starting structures. Each of the docked structures was energy minimized to the stage where the root-mean-square gradient was less than 0.1 kcal/(mol Å). The distance-dependent dielectric constant of $\epsilon = 4r_{ij}$ was used in the calculation of the docked structures. Structural effects of water and counterions were neglected in the energy calculations.

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