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Apoprotein-Assisted Unusual Cyclization of Neocarzinostatin Chromophore

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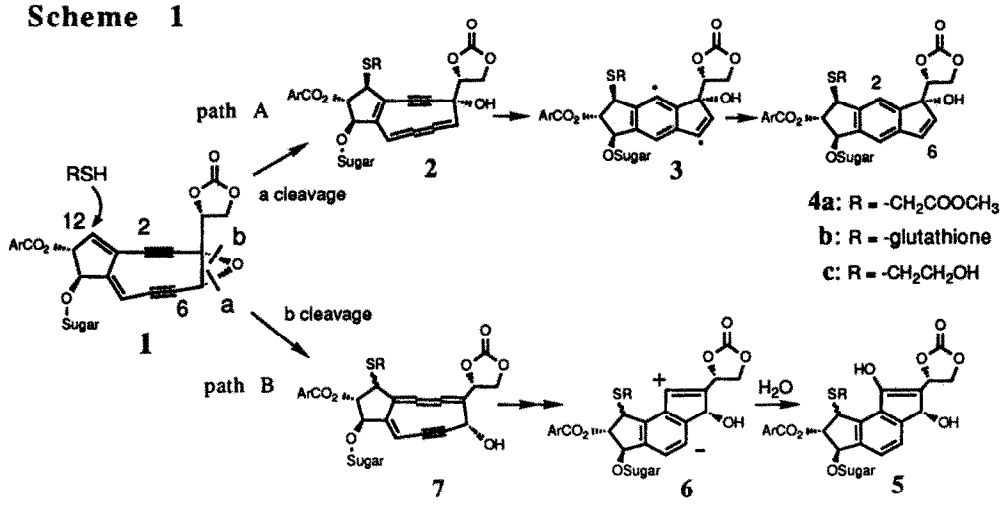
Abstract: *The reaction of neocarzinostatin chromophore (**1**) with 2-mercaptoethanol in the presence and absence of apoprotein was investigated. When complexed with apoprotein, **1** was found to react with 2-mercaptoethanol to provide unprecedented cyclization product **5** as a major product together with a minor amount of known cyclization product **4c**. The structure of **5** was elucidated by spectroscopic data including ^{1}H- and ^{13}C-NMR, FABMS, and FTIR. Addition of isopropanol to the reaction system dramatically changed the ratio of **5** to **4c**. In a Tris-HCl buffer containing 80% isopropanol, **4c** was formed as a major product together with a minor amount of **5**. The reaction of isolated pure **1** with 2-mercaptoethanol in Tris-HCl buffer containing isopropanol gave **4c** as a major product together with trace of **5**. These results indicate that **1** shows a quite different reactivity toward thiols when complexed with apoprotein: nucleophilic attack of the thiol at C12 of **1** complexed with apoprotein gives a different type of enyne[3]cumulene **7** which undergoes cycloaromatization followed by hydration to provide **5**. The fact that the binding constant of **5** toward apoprotein is one order of magnitude larger than that of **4c** suggests that the stability of the apoprotein-**5** complex may be closely related to the preferential formation of **5** in an appoprotein complex.*

Neocarzinostatin (NCS) is an antitumor antibiotic consisting of nonprotein chromophore **1** and its carrier apoprotein.[1] Antitumor activity of NCS is believed to be due to its strong DNA cleaving activity. Since the DNA cleavage induced by NCS complex is largely reproducible by isolated NCS chromophore **1**,[2] the reaction of purified **1** with a variety of thiols in organic solvents has been investigated extensively.[3] On the basis of the structural elucidation of methyl thioglycolate-**1** adduct (**4a**) as well as on the observation of enyne[3]cumulene intermediate **2a** by low temperature ^{1}H NMR,[3c] the activation mechanism of NCS chromophore **1** has been proposed as shown in Scheme 1 (path A): nucleophilic attack by thiol at C_{12} and epoxide ring opening would generate **2** which spontaneously cyclizes to form the indacene diradical **3**. This diradical species **3** has been proposed to abstract hydrogen from DNA deoxyribose backbone to induce DNA strand cleavage in the presence of oxygen. In fact, Goldberg *et al.*, have recently demonstrated that ribose C5' deuterium of T_8 of $d(GCAGCGCTGC)_2$ was actually incorporated into C6 position of **4b** (44 %D) in the cleavage reaction using glutathione as a thiol activator.[4] More recently the same group has demonstrated that ribose C1' deuterium of C_5 of $d(GCAGCICTGC)_2$ was incorporated into C2 position of **4b** (22 %D).[5]

In recent years we have been investigating NCS-mediated DNA strand cleavage reactions by utilizing various synthetic oligonucleotides as DNA substrates to address chemical basis for the oxidative DNA strand cleavage initiated by hydrogen abstraction.[6] We found that thiol-activated NCS competitively abstracts C4' and C5' hydrogens in a sequence dependent manner and have proposed a binding model of thiol-activated **1** to duplex DNA on the basis of detail analysis of DNA cleavage data.[6d] While isolated pure **1** shows the same sequence specificity for DNA cleavage as that of the compete NCS complex, the extent of strand cleavage per drug was less than 10 % of the complete system.[7] Therefore, in our investigation on the NCS-mediated DNA cleavage reaction we have employed complete NCS complex containing both apoprotein and **1** in order to obtain sufficient DNA cleavage products enough to allow their full structural elucidation. During these studies, we found that the reaction of NCS complex with 2-mercaptoethanol gives an unusual cyclization product, **5**, as a major product together with known type of cyclization product **4c** as a minor product (path B).[8] The former process proceeded efficiently only when **1** was complexed with apoprotein and has proved to be a major inactivation path for **1** under physiological conditions.

Scheme 1



RESULTS AND DISCUSSION

Isolation and structural elucidation of cyclization products. While the cycloaromatization of **1** providing indene **4a** in organic solvents such as tetrahydrofuran-acetic acid has been extensively investigated, the exact nature of thiol-**1** reaction under physiological conditions, *e. g.*, in an aerated aqueous buffered solution, has still remained to be clarified. Under certain aerobic conditions the reaction of **1** with methyl thioglycolate was reported to give oxygenated cyclization products.[9] Thus, we have examined the reaction of **1** with thiols in the presence of apoprotein under the conditions where DNA cleavage experiments are usually conducted. A solution of lyophilized NCS powder (0.25 mM) and 2-mercaptoethanol (4 mM) in 50 mM Tris-HCl (pH 7.0) was incubated at 0 °C under aerobic conditions. Under these conditions DNA substrate d(GCATGC)2 was degraded to d(GCA)p and 5'-aldehyde fragment d(T*GC) as reported previously.[6a] HPLC analysis of the reaction mixture after 12 h incubation revealed the formation of a major product eluted at 11.0 min together

with the known type of cyclization product **4c** at 8.8 min and other minor products (Figure 1 (B)). The major product was isolated by HPLC, and its structure was assigned as **5** on the basis of following spectral data. FAB mass data (M+1, 756) indicated that the product is a 1:1 adduct between **1** and 2-mercaptoethanol with an additional incorporation of one molecule of water. The FTIR spectrum showed that both carbonate (1805 cm^{-1}) and naphthoate (1645 cm^{-1}) carbonyl groups are preserved in the product.

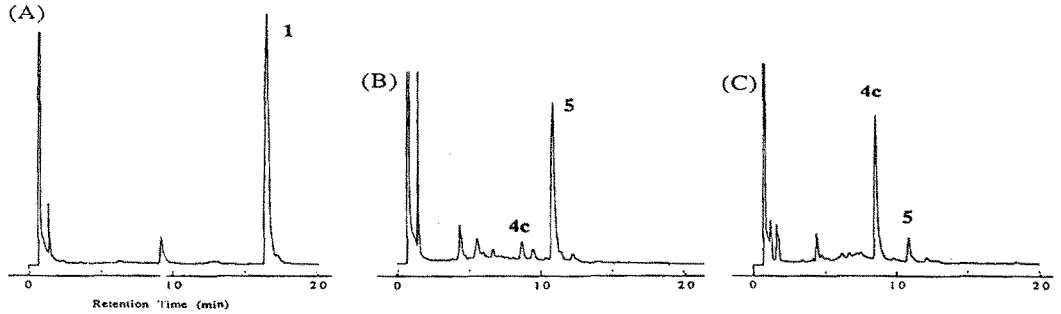


Figure 1. HPLC profiles of (A) NCS, (B) the reaction mixture of NCS and 2-mercaptoethanol in Tris-HCl buffer (pH 7.0) after 12 h incubation, and (C) the same reaction as (B) in a buffer containing 80% isopropanol.

Figure 2 showed the ^1H-^1H COSY (A) and NOESY (B) spectra of the adduct **5** observed in DMSO-d_6 at 400 MHz. The ^1H-^1H COSY NMR spectrum indicates the presence of naphthoate, *N*-methylfucosamine and carbonate subunits, together with ethylene unit of 2-mercaptoethanol. In addition to these readily assignable signals, eight protons (two aromatic, four nonaromatic and two exchangeable protons) attributable to the rearranged core unit were observed. The signals assigned as C5, C10, C11, and C12 protons of the core unit appeared as a set of pair peaks for each proton. These results suggest that the product is a 1 : 1 diastereomeric mixture probably at C12. The most characteristic signals of the product were two doublets (δ 6.99, 7.23) in the aromatic region coupled each other with a coupling constant of 8.2 Hz which were assigned as C7 and C8, respectively. In CD_3OD a pair of doublet aromatic protons of the naphthoate group (3" and 4") together with a pair of doublet aromatic protons (C7 and C8) were well resolved as shown in Figure 3. The COSY experiment revealed a coupling pathway linking five protons of the core unit (C7-C8-C10-C11-C12). The nonaromatic two singlets at δ 5.99 and 6.00 ppm were assigned as C5 proton of the diastereomers which are coupled with exchangeable 5OH proton (δ 7.47, and 7.60). Other exchangeable signals (one proton) appeared at 7.22 and 7.26 ppm were assigned as 3OH of the diastereomers each of which has a long range couplings with C13 proton and shows NOE with C12, C13, and one of C14 methylene protons. The NOE data strongly suggest the intramolecular hydrogen bonding of 3OH with oxygen atom of the carbonate moiety, resulting in a stabilization of an unusual enol form for **5** as shown in Scheme 2. In fact, the deuterium exchange rate of 3OH signals was relatively slow ($t_{1/2}$ > 2h) in DMSO-d_6 compared to that for another exchangeable protons such as 5OH ($t_{1/2}$ < 1 min). Table 1 lists the full assignment of ^1H-^1H COSY and NOESY crosspeaks for **5**. These data together with ^{13}C-^1H COSY (data not shown) demonstrate that the product is a 1 : 1 mixture of diasteromers at C12 and allow complete assignment of all of the ^1H and ^{13}C signals as summarized in Table 2. Unfortunately, attempts to separate these diastereomers under various HPLC or TLC conditions were unsuccessful.

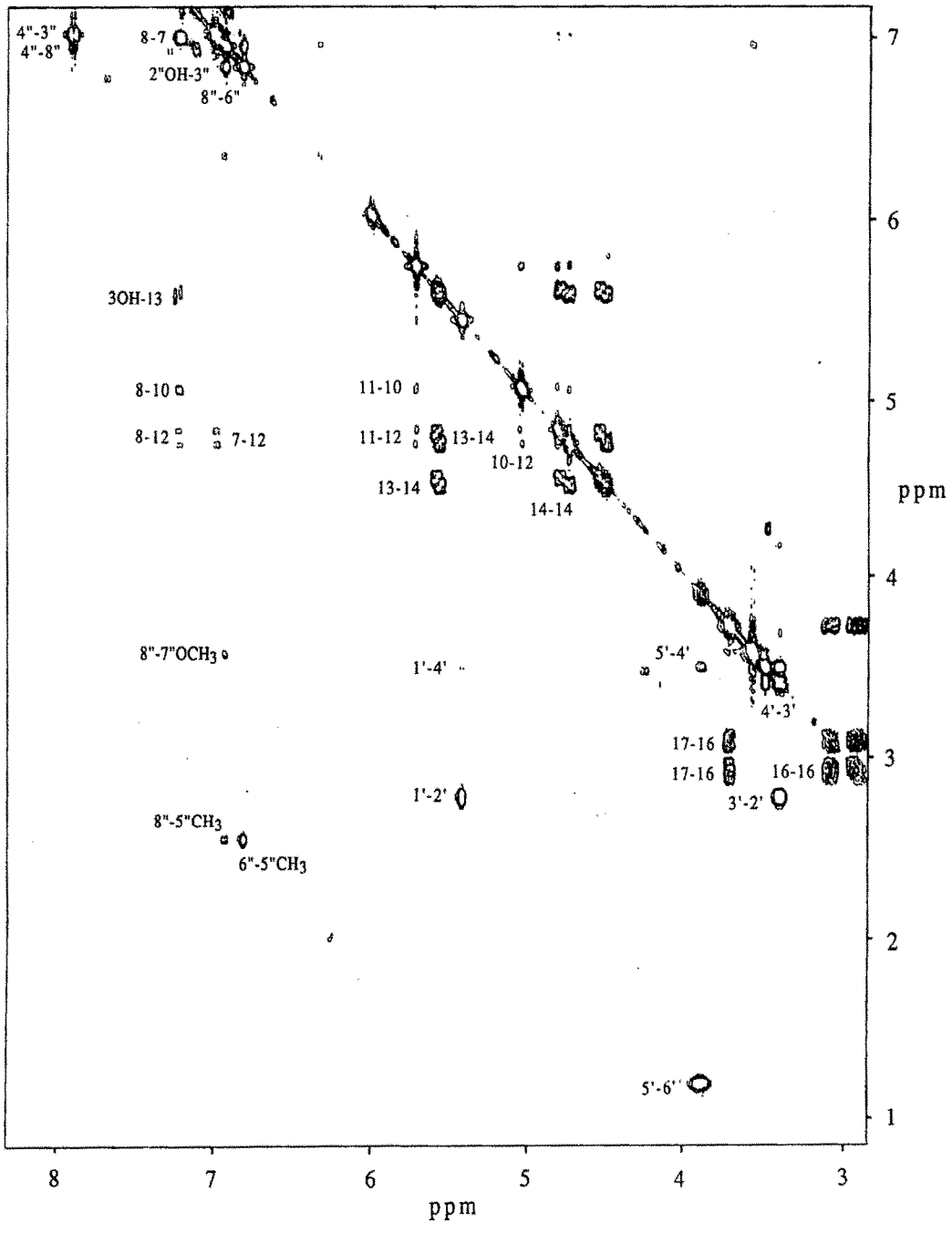


Figure 2(A). ^1H-^1H COSY spectrum of **5** in DMSO-d_6 at 30 °C. Crosspeaks are labeled according to their chemical shifts along ω_1(vertical axis) and ω_2 (horizontal axis).

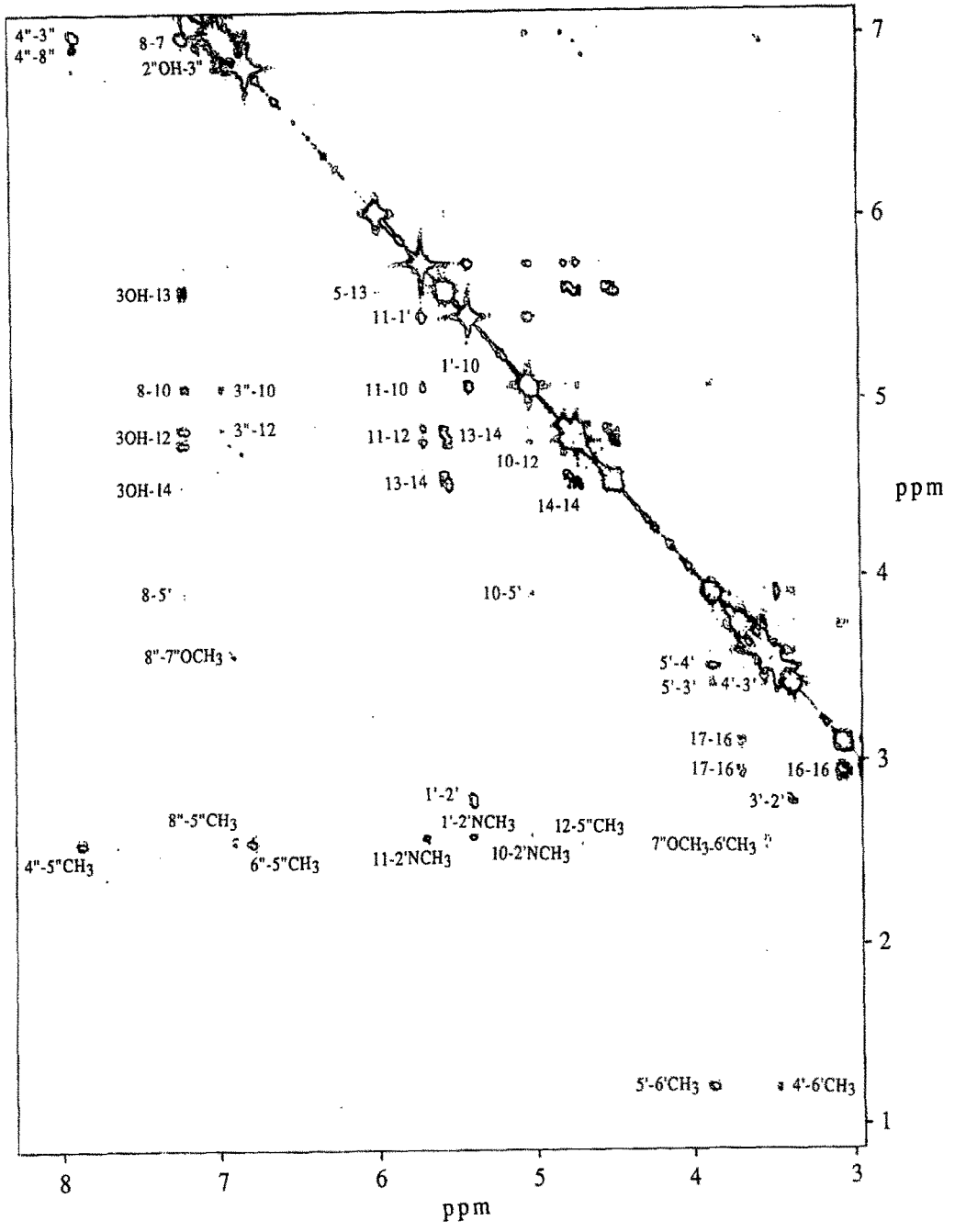


Figure 2(B). NOESY spectrum of **5** in DMSO-d_6 at 30 °C with 300 msec mixing time. Crosspeaks are labeled according to their chemical shifts along ω_1(vertical axis) and ω_2 (horizontal axis).

Scheme 2

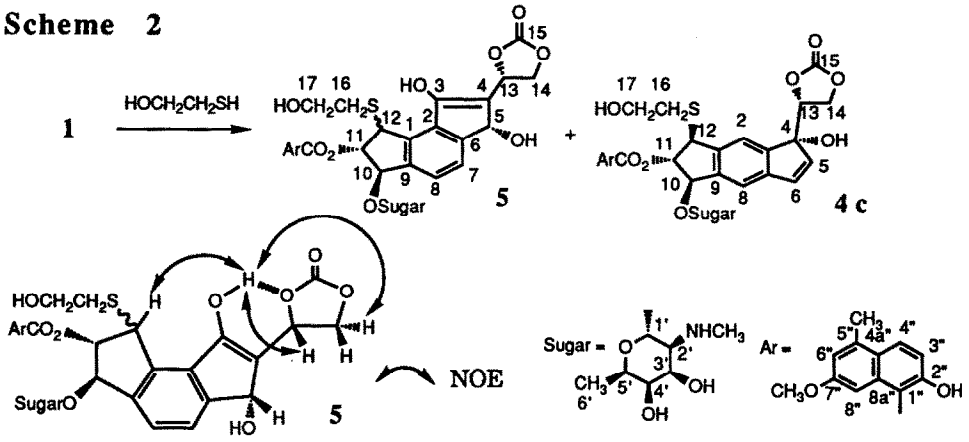


Table 1. Assignment of 2D NMR Cross Peaks (^{1}H-^{1}H COSY) for **5**

cross peak	COSY	NOESY
1	4"-3"	4"-3"
2	4"-8"	4"-8"
3	8-7	4"-5"CH_3
4	3OH-13	8-7
5	8-10	3OH-13
6	8-12	8-10
7	2"OH-3"	3OH-12
8	7-12	3OH-14
9	8"-6"	8-5'
10	8"-7"OCH_3	2"OH-3"
11	8"-5"CH_3	3"-10
12	6"-5"CH_3	3"-12
13	11-10	8"-7"OCH_3
14	11-12	8"-5"CH_3
15	13-14	6"-5"CH_3
16	13-14	5-13
17	1'-4'	11-1'
18	1'-2'	11-10
19	10-12	11-12
20	14-14	11-2'NCH_3
21	5'-4'	13-14
22	5'-6'	13-14
23	17-16	1'-10
24	17-16	1'-2'
25	4'-3'	1'-2'NCH_3
26	3'-2'	10-12
27	16-16	10-5'
28		10-2'NCH_3
29		14-14
30		12-5"CH_3
31		5'-4'
32		5'-3'
33		5'-6'CH_3
34		17-16
35		17-16
36		4'-3'
37		7"OCH_3-6'CH_3
38		4'-6'CH_3
39		3'-2'
40		16-16

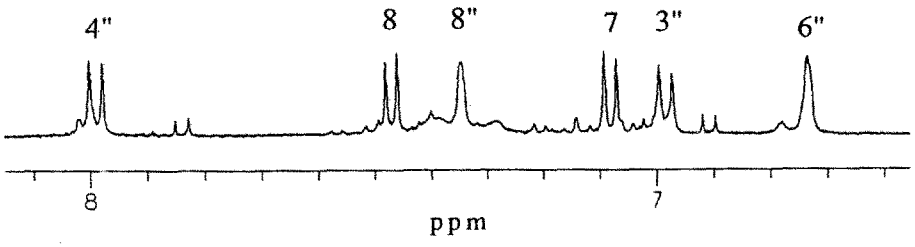


Figure 3. Expansion of the aromatic region of ^1H NMR spectra of **5** in CD_3OD at 24.0 °C.

Table 2. Assignment of ^1H (400 MHz) and ^{13}C (100 MHz) NMR Data for **5** in DMSO-d_6

Assignment	^{13}C (ppm)	^1H(ppm)
1	128.4(s), 129.5(s)*	
2	134.5(s), 135.3(s)*	
3	139.2(s), 139.9(s)*	
4	132.9(s)	
5	88.1(d), 89.0(d)*	5.99, 6.00* (1H, s)
6	156.2(s), 156.3(s)*	
7	117.7(d), 117.8(d)*	6.99 (d, 1 H, J = 8.2 Hz)
8	127.2(d), 127.3(d)*	7.23 (d, 1 H, J = 8.2 Hz)
9	138.2(s), 138.9(s)*	
10	82.1(d)	5.05, 5.03* (bs, 1 H)
11	84.2(d), 84.4(d)*	5.72, 5.71* (bs, 1 H)
12	50.3(d), 50.4(d)*	4.81(b), 4.73(a)* (bs, 1 H)
13	76.1(d), 76.6(d)*	5.57 (dd, 1 H, J = 16.0, 8.1 Hz)
14	68.6(t)	4.53 (dd, 1 H, J = 16.5, 7.9 Hz), 4.73 (dd, 1 H, J = 16.5, 6.9 Hz)
15	154.6(s), 154.7(s)*	
16	34.6(t), 34.8(t)*	3.06 (m, 1 H), 2.98 (m, 1 H)
17	61.0(t)	3.70, 3.71* (t, 2 H, J = 7.0 Hz)
1'	96.2(d), 96.3(d)*	5.42 (bs, 1 H)
2'	58.1(d)	2.75 (d, 1 H, J = 11.0 Hz)
3'	68.9(d)	3.38 (d, 1 H, J = 11.0 Hz)
4'	70.8(d)	3.48 (bs, 1 H)
5'	66.8(d)	3.90 (t, 1 H, J = 7.9 Hz)
6'	16.8(q)	1.17 (d, 3 H, J = 6.4 Hz)
2'NCH_3	33.9(q)	2.55 (s, 3 H)
1"	110.9(s), 111.1(s)*	
2"	168.2(s)	
3"	115.3(s)	7.01 (d, 1 H, J = 9.2 Hz)
4"	129.3(d)	7.91 (d, 1 H, J = 9.2 Hz)
5"	136.8(s)	
6"	116.6(s)	6.82 (s, 1 H)
7"	158.4(s)	
8"	100.5(d)	6.98 (s, 1 H)
4a"	120.1(s)	
8a"	133.4(s)	
1"CO_2	172.6(s)	
5"CH_3	19.3(q)	2.52 (s, 3 H)
7"OCH_3	54.6(q), 54.8(q)*	3.55, 3.57* (s, 3H)
3OH		7.22, 7.26* (s, 1 H)
5OH		7.47, 7.60* (br, 1H)
2"OH		7.12 (br, 1 H)
17OH, 2'NH, 3'OH, 4'OH		not identified

* Peaks due to the diastereomer at C12.

The major factor controlling two cycloaromatization processes (path A and path B) is whether the bond a or b of the epoxide ring is cleaved in the thiol addition reaction (Scheme 1). While the exact reason for the predominant formation of **5** in apoprotein is not clear, it is apparent that the cleavage of epoxide b bond is facilitated only in an apoprotein complex. The fact that binding affinity of **5** to apoprotein (2.5×10^7) is one order of magnitude larger than that of **4c** (2.1×10^6) would suggest that the properties of apoprotein interiors such as hydrogen bonding ability or steric interaction may play an important role in controlling the direction of ring opening of the strained epoxide. Nonstereospecificity for the thiol addition at C12 observed in the formation of **5** would be explained in terms of the lack of assistance of *N*-methylfucosamine moiety in an apoprotein complex in the nucleophilic attack of thiols, which has been proposed recently for the stereospecific formation of **4a**.[10]

Proposed mechanism for the formation of 5. The cycloaromatization of **1** leading to **5** occurs almost equally either under O_2 or Ar atmosphere, suggesting that molecular oxygen is not incorporated into **5**. In order to elucidate the origin of two hydrogens and one oxygen incorporated into **5**, the reaction of NCS complex with 2-mercaptoethanol was performed in $H_2{}^{18}O$- or D_2O-containing buffer. In each reaction the product was isolated by HPLC and subjected to spectroscopic analyses. When the reaction was carried out in $H_2{}^{18}O$ (99.0 atom %), the parent (M+1) FABMS peak of the product was shifted to 758, indicating the incorporation of one ^{18}O atom into **5**. When D_2O (99.9%D) was used as solvent, 80% of the deuterium was incorporated into the C_7 position of **5d** as determined by ^1H NMR (Figure 4). These results strongly suggest that **5** is formed via hydration of a zwitterionic precursor such as **6** (Scheme 3). An analogous polar addition of nucleophilic solvent has been reported in the cyclization of 1,2,4-heptatrien-6-yne.[11] Alternatively, it seems not implausible that direct nucleophilic attack of water to strained enyne[3]cumulene **7** would produce **5** via **8**.[12] The possibility of such diverse modes of cycloaromatization of enyne systems has been proposed in the reaction of NCS model compounds.[13] Present results demonstrated for the first time that there are two distinct cycloaromatization pathways in the reaction of **1** with thiol in an apoprotein complex.

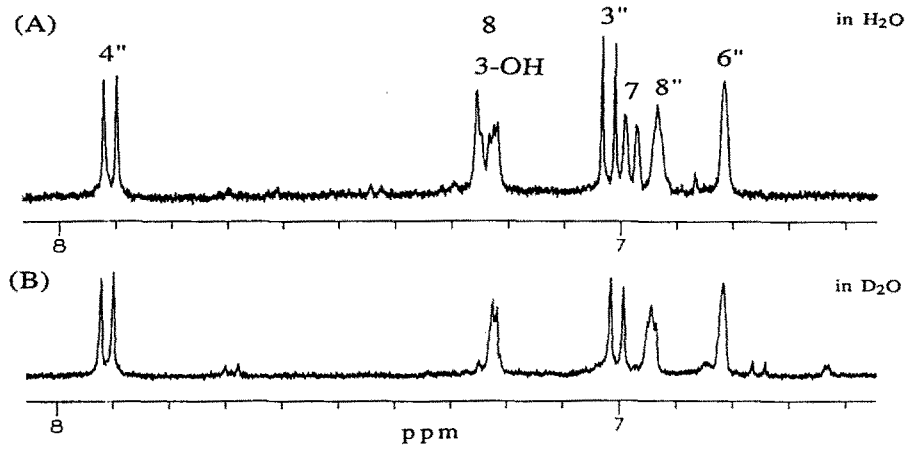


Figure 4. Partial ^1H NMR spectra (DMSO-d_6, 24.5 °C) of **5** isolated from the reactions in H_2O (A) and D_2O (B).

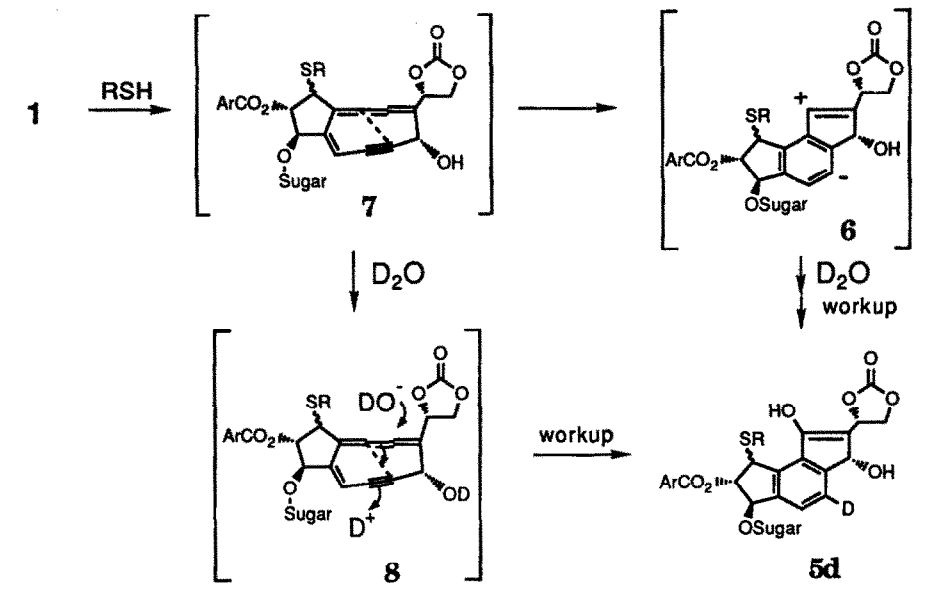
Formation of 4c and 5 under various conditions. A small HPLC peak at 8.8 min in Figure 1 (B) was found to be a major product in the reaction in a solvent containing 80% isopropanol as shown in Figure 1 (C). The product was isolated by means of HPLC, and the structure was assigned as **4c** by comparison of its ^{1}H NMR spectrum with the reported data for **4a** together with FABMS (M+1, 740) data. In contrast to the formation of a 1:1 diastereomeric mixture for **5**, nucleophilic attack of thiol at C12 of **1** occurred in a stereospecific manner to give **4c** as a single isomer in accord with the previous result on the formation of **4a**.[3b] Quantitative analysis of the reaction mixture indicates that the yields of **5** and **4c** in aqueous buffer solution without isopropanol are 59% and 9.5%, respectively. These results indicate that a major reaction between **1** and 2-mercaptoethanol in an apoprotein complex is the formation of a different type of cyclization product, **5**. Interestingly, the ratio of **5** *vs.* **4c** was found to be highly dependent on the content of isopropanol in the reaction system. For instance, in a solvent containing 80% isopropanol **4c** (45%) was a major product together with a minor amount of **5** (5.1%). A similar solvent effect on the ratio of **5** *vs.* **4c** was observed by using other solvents such as *t*-butanol. Since **1** is extractable from NCS complex with organic solvents such as acidic methanol or acetic acid-tetrahydrofuran, it is very likely that addition of a large amount of isopropanol to the reaction mixture would result in a liberation of **1** from NCS complex and, as a result, change the product ratio. In fact, **1** extracted from NCS complex by reported procedure[3b] was found to react with 2-mercaptoethanol in isopropanol to provide **4c** as a sole detectable product. However, in aqueous buffer solution extracted **1** gave neither **5** nor **4c** in the absence of isopropanol to result in a formation of a complex mixture of multiple products. This decomposition may probably be due to the complicated intra- or intermolecular hydrogen abstraction reactions by biradical **3** in the absence of excess isopropanol as hydrogen donor. Addition of isopropanol to the reaction system dramatically increased the yield of cyclization product **4c**, indicating that for the efficient formation of **4c** the presence of isopropanol as external hydrogen donor is indispensable. The ratios of **5** *vs.* **4c** at different concentrations of isopropanol were summarized in Table 3. These results suggest that **5** is produced only when **1** is complexed with apoprotein in aqueous solution. The reason for the lack of previous reports on the formation of **5** is probably due to the extremely low yield of **5** without apoprotein.

Table 3. Effect of the addition of isopropanol on the formation of **4c** and **5** in the reaction of **1** with 2-mercaptoethanol in Tris-HCl buffer

isopropanol(%)	NCS complex (**1**+apoprotein)		**1** only	
	4c(%)	**5**(%)	**4c**(%)	**5**(%)
0	9.5	50	0	0
4	nd	nd	5.6	0.2
10	15	46	43	2.8
80	45	5.1	nd	nd

nd: not determined.

Scheme 3



Possible role of path B giving 5 in DNA cleavage. In order to know the possible role of **5** in the DNA cleavage process, the incorporation of tritium into **5** from [2,8,5'-^{3}H]-labeled poly(dAdT) was examined. Figure 5 showed the HPLC profile of the cleavage of tritium-labelled poly(dAdT) by 2-mercaptoethanol-activated NCS. In contrast to the significant incorporation of tritium into **4c**, almost no incorporation into **5** was observed. These results clearly indicate that the process giving **5** is not directly involved in the DNA cleavage reaction, suggesting that this cycloaromatization (path B) is an inactivation process for **1** in an apoprotein complex.

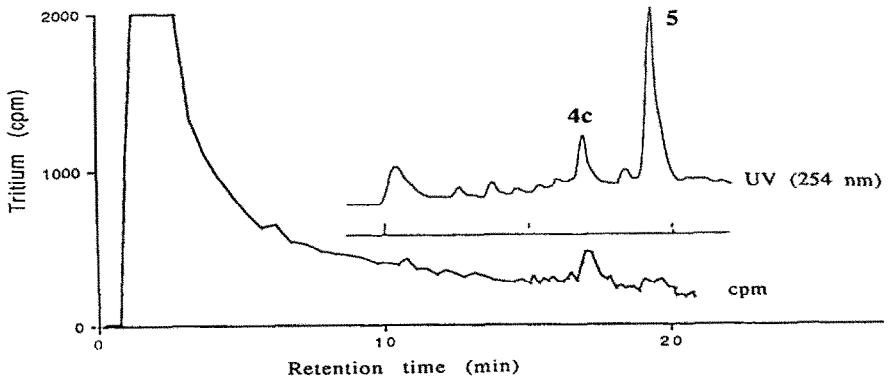


Figure 5. HPLC profiles of the reaction mixture of NCS and 2-mercaptoethanol in the presence of poly([2,8,5'-^{3}H]dAdT). Detections were carried out by radioactivity (^{3}H) and UV absorbance at 254 nm (inset).

It is generally believed that NCS apoprotein has an important function to protect thermally labile and light sensitive chromophore **1** against its decomposition. The present work, however, has demonstrated that NCS apoprotein has an alternative function to facilitate an inactivation process for **1** giving a non DNA-damaging cyclization intermediate. It should be noted here that the rate of DNA strand cleavage decreased considerably in the presence of apoprotein, *i.e.*, the DNA cleavage was completed within 30 min by **1**, whereas NCS complex required 12 h for the same DNA cleavage. Figure 6 showed the relative DNA-cleaving activity of NCS-apoprotein complex in the presence of various concentrations of *t*-butanol. The cleavage increased dramatically with increasing concentration of *t*-butanol; in 25% *t*-butanol the amount of DNA cleavage was 2.5 times enhanced compared to the control. In contrast, such enhancement was not observed for Fe-bleomycin (BLM)-mediated reaction (data not shown) which is known to induce DNA cleavage via deoxyribose C4' hydrogen abstraction.[15] These results indicate that addition of organic solvents such as *t*-butanol to the reaction system results in a release of **1** from NCS complex to reduce the portion of the inactivation process giving **5** and, as a result, enhances the relative DNA-cleaving activity. Although a similar enhancement of DNA-cleaving activity by addition of organic solvents was previously observed by Goldberg et al.,[16] the exact reason for this phenomena has not been fully understood.

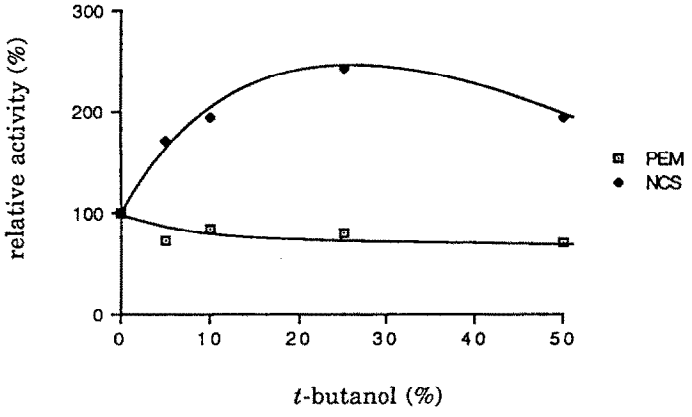


Figure 6. Relative DNA-cleaving activity of NCS (•) and peplomycin (▫) at different concentrations of *t*-butanol. DNA-cleaving activity was estimated by the amount of free bases produced from $d(GCATGC)_2$ after alkaline treatment (0.1 N NaOH, 90 °C, 5 min). The relative activity was calculated on the basis of the control run without *t*-butanol as a strandard.

In conclusion, the present work has demonstrated that there are two distinct cycloaromatization pathways in the reaction of **1** with 2-mercaptoethanol in NCS complex. The new pathway leading to **5** was proven to be a major inactivation path for **1** in NCS apoprotein complex.

EXPERIMENTAL

Materials and Methods. Neocarzinostatin complex (NCS) and its apoprotein were obtained from Pola Kasei Co. Ltd. The concentration of NCS chromophore was determined spectrophotometrically (ε_{340} 10,800).[17] Concentration of apoprotein was determined from the absorption at 227 nm (ε_{227} 14000).[18] $H_2{}^{18}O$ (99.0 atom %) was purchased from ISOTEC Inc. Calf intestine alkaline phosphatase (AP, 1000 unit/mL) and snake venom phosphodiesterase (s.v. PDE, 3 unit/mL) were purchased from Boehringer Mannheim. E. coli DNA polymerase I-large fragment (Klenow fragment, 2 unit/μL) was purchased from Takara Shuzo Co., Ltd. Poly(dAdT) was obtained from Pharmacia. [2,8,5'-^{3}H]dATP tetrasodium salt (1.25-0.85 mM, specific activity 40-60 Ci/mmol) was obtained from Dupont (NEN Research Products). SEP-PAK C18 cartridge was purchased from Waters. d(GCATGC)$_2$ was prepared by the β-(cyanoethyl)phosphoramidite method[19] on controlled pore glass supports (1 mmole) by using ABI 381 A DNA synthesizer. The 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 d(GCATGC)$_2$ were determined by complete digestion with s.v. PDE and AP to 2'-deoxymononucleosides. Silica gel column chromatography was carried out on Wakogel C-200. Preparative TLC was carried out on Merck silica gel 60 PF$_{254}$ plate. ^{1}H NMR spectra were recorded on a JEOL-JNM-GX400 spectrometer.

Isolation of **5**. Neocarzinostatin powder (0.5 g, 0.04 mmol) was dissolved in 20 mL of ice-cooled Tris-HCl (250 mM, pH 7.0) buffer (20 mL). To this solution was added 2-mercaptoethanol (286 mg, 3.7 mmol), and the mixture was stirred at 0 °C for 12 h under dark. The resulting solution was subjected to HPLC (μBondasphere 5C$_{18}$ (Waters); 0.05 M ammonium acetate (pH 4.0) containing 30-50% acetonitrile, for 20 min; flow rate 10 mL/min; retention time, 13 min). Fractions containing **5** (total volume: 30 mL) were combined and concentrated to a volume of 15 mL. The solution was loaded on SEP-PAK cartridge and washed with 50 mL of distilled water. **5** was eluted with 10 mL of methanol - water (1 : 1) and methanol was removed under reduced pressure. After lyophilization 9 mg (30%) of **5** was obtained: FABMS positive 756 (M+1); UV (CH_3OH) 340 nm (ε 15700); ^{1}H-^{1}H COSY and NOESY were shown in Figure 2.

Isolation of **4c**. Neocarzinostatin powder (0.75 g, 0.06 mmol) was dissolved in 20 mL of ice-cooled Tris-HCl (250 mM, pH 7.0) buffer containing 80% of isopropanol. To this solution was added 2-mercaptoethanol (286 mg, 3.7 mmol), and the mixture was stirred at 0 °C for 12 h under dark. Isopropanol was removed under reduced pressure and the resulting solution was subjected to HPLC (μBondasphere 5C$_{18}$ (Waters); 0.05 M ammonium acetate (pH 4.0) containing 30-50% acetonitrile/20 min; flow rate 10 mL/min; retention time, 10.5 min). After desalting by SEP-PAK cartridge followed by lyophilization, 8 mg (18%) of **4c** was obtained. **4c**: ^{1}H NMR (DMSO-d$_6$) δ 1.18 (d, 3 H, J = 6.5 Hz, 6"), 2.51 (s, 3 H, 2'NCH$_3$), 2.54 (s, 3 H, 5"CH$_3$), 2.76 (m, 2 H, 16, 2'), 2.87 (dt, 1 H, J = 12.8, 6.5 Hz, 16), 3.50 (d, 1 H, J = 3.3 Hz, 3'), 3.53 (brs, 1 H, 4'), 3.62 , 3.63 (t, 2 H, J = 7.1 Hz, 17), 3.72 (s, 3 H, 7"OCH$_3$), 4.00 (q, 1 H, J = 6.4 Hz, 5'), 4.37 (dd, 1 H, J = 5.5, 2.9 Hz, 14), 4.47 (d, 1 H, J = 3.0 Hz, 12), 4.48 (t, 1 H, J = 5.5 Hz, 13), 4.63 (dd, 1 H, J = 5.5, 2.9 Hz, 14), 5.19 (d, 1 H, J = 2.8 Hz, 10), 5.48 (d, 1 H, J = 3.7 Hz, 1'), 5.76 (dd, 1 H, J = 3.0, 2.8 Hz, 11), 6.32 (d, 1 H, J = 5.7 Hz, 5), 6.85 (s, 1 H, 6"), 6.94 (d, 1 H, J = 5.7 Hz, 6), 7.02 (d, 1

H, J = 9.0 Hz, 3"), 7.23 (s, 1 H, 8), 7.54 (s, 1 H, 2), 7.90 (d, 1 H, 9.0 Hz, 4"). FABMS positive 740 (M+1). UV (CH_3OH) 340 nm (ε 14800).

Incorporation of deuterium from D_2O into 5 and 4c. Neocarzinostatin powder (0.37 g, 0.03 mmol) was lyophilized twice from 10 mL of D_2O. Neocarzinostatin powder was then dissolved in 20 mL of ice-cooled Tris-DCl (250 mM, pD 7.4) buffer. To this solution was added deuteriated 2-mercaptoethanol (286 mg, 3.7 mmol), and the mixture was stirred at 0 °C for 12 h under dark. The resulting solution was subjected to HPLC (μBondasphere $5C_{18}$ (Waters); 0.05 M ammonium acetate (pH 4.0) containing 30-50% acetonitrile/linear gradient 20 min; flow rate 10 mL/min). After desalting by SEP-PAK cartridge followed by lyophilization, **5** (0.3 mg) and **4c** (0.1 mg) were obtained. Each sample was subjected to ^1H NMR analysis.

Incorporation of ^{18}O from $H_2{}^{18}O$ into 5. Neocarzinostatin powder (15 mg, 1.2 mmol) was dissolved in 500 mL of $H_2{}^{18}O$ (99.0 atom % enriched). The solution pH was adjusted to pH 7.0 with 250 mM Tris-HCl. To this solution was added 2-mercaptoethanol (2.9 mg, 0.037 mmol), and the mixture was stirred at 0 °C for 12 h under dark. **5** was isolated as described above and subjected to FABMS.

Formation of 4c and 5 under various conditions. The reaction mixtures (total volume 100 μL) containing NCS (0.25 mM), 2-mercaptoethanol (4 mM) and indicated amounts of isopropanol in 50 mM Tris-HCl (pH7.2) were incubated under dark at 0 °C for 12 h. 10 mL of aliquot was subjected to HPLC. Analysis was carried out on Wakosil $5C_{18}$ column (4.6 x 150 mm). Elution was with 0.05 M ammonium acetate containing 30-50% acetonitrile/20 min linear gradient at a flow rate of 1.5 mL/min.

Incorporation of tritium into 5 and 4c. Poly([2,8,5'-^{3}H]dAdT) was prepared according to the modification of the reported procedure.[20] The reaction mixture (500 μL) containing E. coli DNA polymerase I-large fragment (Klenow fragment, 12 unit), poly(dAdT) (0.25 mM), [2,8,5'-^{3}H]dATP, tetrasodium salt (1.25-0.85 mM, specific activity of 40-60 Ci/mmol), dATP (0.3 mM), TTP (0.3 mM), and magnesium acetate (5 mM) in 100 mM Tris-acetate (pH 7.0) was incubated at 37 °C for 18 h. The reaction mixture was transfered to a sample cup of Ultrafree C3 and was centrifuged at 2000G for 8 min. The supernatant was washed with three times with 500 μL of water. The specific activity of the product was 6.7 x 10^{10} cpm/mmol of mononucleotide. Reaction mixtures (total volume 75 μL) containing poly(dAdT) (0.2 mM, base concentration) and NCS (0.25 mM) in 50 mM Tris-HCl (pH 7.0) were prepared. To this reaction mixture was added 25 μL of 2-mercaptoethanol solution to initiate the reaction. After incubation for 12 h at 0 °C under aerobic conditions, 10 μL of the aliquot was subjected to HPLC analysis on a Cosmosil $5C_4$ column (4.6 x 150 mm). Elution was with 0.05 M ammonium acetate containing 20-40% acetonitrile/20 min linear gradient at a flow rate of 1.5 mL/min. The fractions were collected (0-10 min: 30 sec, 10-15 min: 20 sec, 15-20 min: 10 sec, 20-22 min: 20 sec). Radioactivity was determined by using a toluene-based scintillation cocktail and calibrated with a volume of fraction.

Determination of binding constant. The binding constants for **4c** and **5** to NCS apoprotein were determined by the reported procedure.[21] Small volume of apoprotein solutions, covering a wide range of

concentrations, were added to 50 μM of **5** or **4c** in 5 mM Tris-HCl buffer (pH 7.0) containing 5% of methanol. The fluorescent intensities were measured at 440 nm under excitation at 340 nm. The binding constants of **4c** and **5** were determined as 2.1 x 10^6 and 2.5 x 10^7, respectively.

Cleavage of d(GCATGC)$_2$ by NCS in the presence of t-butanol. Each of the reaction mixture (total volume 100 μL) contained hexamer (1 mM), 2-mercaptoethanol (4 mM), and NCS (250 μM) were prepared in 50 mM Tris-HCl (pH 7.2). After incubation at 0 °C for 12 h under aerobic conditions, 2 μL of 1 N NaOH was added, and the solution was heated at 90 °C for 5 min. The resulting mixture was neutralized and then subjected to HPLC analysis. 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 - 15 % acetonitrile, linear gradient (20 min) at a flow rate of 1.5 mL/min. Under this condition C5' cleavage product such as 5'-aldehyde d(T*GC) was quantitatively degraded to thymine.[6a,d] Thus, the total event induced by NCS was estimated as the total amount of thymine.

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Note Added in Proof. Very recently, Chin and Goldberg have also confirmed that treatment of apoprotein-bound NCS-Chromophore with 2-mercaptoethanol produces the same cyclization product **5** and reported that **5** is not involved in the DNA cleavage reaction (Chin, D.-H.; Goldberg, I. H. *J. Am. Chem. Soc.* **1993,** *115,* 9343). Their conclusions were virtually the same as those reported earlier[8] and described in the present full paper. However, concerning the structure of **5**, they suggested that **5** has a hydrogen at C3 rather than a hydroxyl group. As already mentioned in our communication,[8] **5** has a hydroxyl group at C3 which is strongly hydrogen-bonded to an oxygen of the carbonate moiety, thus making very slow exchange of 3OH with D_2O in DMSO-d_6 as described in the present paper.

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