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A Comparison of DNA Cleavage by Neocarzinostatin Chromophore and Its Aglycon: Evaluating the Role of the Carbohydrate Residue

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Abstract: Through a comparative analysis of the reactivity and DNA cleaving activity of neocarzinostatin (NCS) chromophore (**1**) and the corresponding aglycon (**2**), we show that the carbohydrate residue (aminoglycoside) of **1** both accelerates the rate and improves the efficiency of DNA cleavage versus the aglycon (**2**), but does not appear to be a major determinant of the base specificity of DNA cleavage by **1**. This stands in contrast to earlier findings with another enediyne antibiotic, calicheamicin γ_1, where the carbohydrate residue was found to be a major determinant of the sequence specificity of DNA cleavage, in addition to playing a functional role in the reductive activation step. Thiol addition experiments with NCS aglycon (**2**) provide further evidence that the carbohydrate amino group of **1** functions as an internal base in the first step leading to DNA cleavage, thiol activation. Evidence is also presented supporting the proposal that NCS aglycon (**2**) is bound tightly and reversibly by the neocarzinostatin binding protein (apo-NCS) and that this binding stabilizes the aglycon in solution.

A small subset of natural antitumor agents that are capable of covalent modification of nucleic acids have been found to do so by free-radical-based mechanisms.[1] Of these, the vast majority are glycosylated,[2] and a natural question arises as to the possible role of these carbohydrate residues in the nucleic acid recognition and/or damaging events. Herein, we address this question as it concerns the aminoglycoside antibiotic neocarzinostatin, the prototypical "enediyne" antitumor agent.

Neocarzinostatin (NCS, holo-NCS) was the first chromoprotein antitumor antibiotic to be characterized and is composed of a highly reactive aminoglycoside chromophore component (**1**) bound noncovalently to a 113-amino acid carrier protein (apo-NCS).[3,4] The details of chromophore binding to its apoprotein have been established by X-ray crystallography.[5] Goldberg and co-workers showed that both the isolated chromophore (**1**) and holo-NCS bring about the cleavage of double-stranded B-form DNA in the presence of a thiol activating agent and have established that the majority of DNA strand breaks

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(1) (a) Nicolaou, K. C.; Dai, W.-M. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1387. (b) *Nucleic Acid Targeted Drug Design*; Propst, C. L., Perun, T. J., Eds.; Marcel Dekker: New York, 1992. (c) Murphy, J. A.; Griffiths, J. *Nat. Prod. Rep.* **1993**, *10*, 551.

(2) The enediyne antibiotic dynemicin A is a notable exception. For leading references, see: (a) Myers, A. G.; Fraley, M. E.; Tom, N. J.; Cohen, S. B.; Madar, D. J. *Chem. Biol.* **1995**, *2*, 33. (b) Myers, A. G.; Cohen, S. B.; Tom, N. J.; Madar, D. J.; Fraley, M. E. *J. Am. Chem. Soc.* **1995**, *117*, 7574.

(3) Isolation: (a) Shoji, J. *J. Antibiot.* **1961**, *14*, 27. (b) Ishida, N.; Miyazaki, K.; Kumagai, K.; Rikimaru, M. *J. Antibiot.* **1965**, *18*, 68. Characterization: (c) Napier, M. A.; Holmquist, B.; Strydom, D. J.; Goldberg, I. H. *Biochem. Biophys. Res. Commun.* **1979**, *89*, 635. (d) Koide, Y.; Ishii, F.; Hasuda, K.; Koyama, Y.; Edo, K.; Katamine, S.; Kitame, F.; Ishida, N. *J. Antibiot.* **1980**, *33*, 342.

(4) Chromophore structure: (a) Edo, K.; Mizugaki, M.; Koide, Y.; Seto, H.; Furihata, K.; Ōtake, N.; Ishida, N. *Tetrahedron Lett.* **1985**, *26*, 331. Carbohydrate stereochemistry: (b) Edo, K.; Akiyama, Y.; Saito, K.; Mizugaki, M.; Koide, Y.; Ishida, N. *J. Antibiot.* **1986**, *39*, 1615. Chromophore stereochemistry: (c) Myers, A. G.; Proteau, P. J.; Handel, T. M. *J. Am. Chem. Soc.* **1988**, *110*, 7212. (d) Myers, A. G.; Proteau, P. J. *J. Am. Chem. Soc.* **1989**, *111*, 1146.

(5) Kim, K.-H.; Kwon, B.-M.; Myers, A. G.; Rees, D. C. *Science* **1993**, *262*, 1042.

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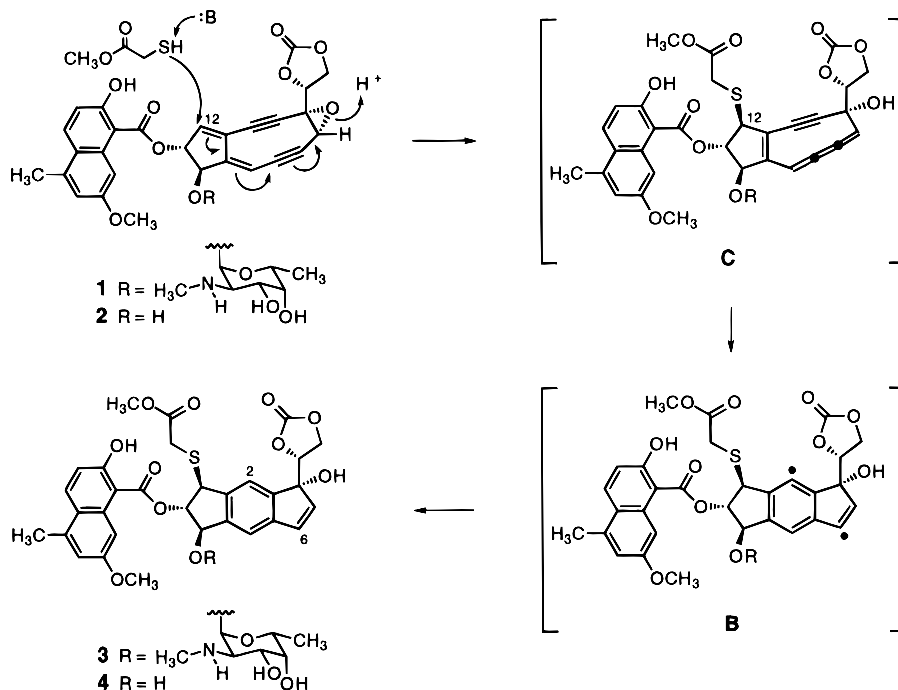
Scheme 1. Proposed Mechanism of Activation of **1** and **2** with Thiols



are single-stranded, arising primarily by the abstraction of a C-5′ hydrogen from thymidylate and adenylate residues by the activated chromophore.[6,7] The pathway shown in Scheme 1 was proposed for chromophore activation[8] and is now supported by a range of experimental evidence.[4d,9] Little sequence specificity is observed in the single-stranded cleavage of DNA by **1** and thiols, but a pronounced base specificity (T > A ≫ C > G) has been established.[10] An important subset of DNA lesions is known to be made up of double-stranded cuts. These exhibit modest sequence specificity over a 3-base-pair range.[11] On the basis of a variety of physical data,[12] Goldberg et al. have proposed a model for chromophore binding that involves intercalation of the naphthoate residue with simultaneous positioning of the glycosylated chromophore core within the minor groove of DNA.[13] From an NMR study of the adduct formed from **1** and glutathione bound to double-stranded DNA, they further proposed that the amino sugar component makes specific contacts with a T•A base pair, contributing to the base specificity of DNA cleavage by **1**.[14] In the present work, through a comparative analysis of the reactivity and DNA cleaving activity of **1** and the corresponding aglycon (**2**), we show that the carbohydrate residue (aminoglycoside) of **1** both accelerates the rate and improves the efficiency of DNA cleavage versus the aglycon (**2**), but does not appear to be a major determinant of the base specificity of DNA cleavage by **1**.

Thiol Addition to NCS Aglycon (2). We recently described an enantioselective, multistep synthetic route to neocarzinostatin chromophore aglycon (**2**).[15] The aglycon (**2**) is found to be much less stable than **1** both in solution and, especially, in neat form. Freshly purified solutions of **2** in organic solvents such as acetonitrile and tetrahydrofuran exhibit varying stabilities, typically on the order of a few hours at 23 °C under an inert atmosphere, although occasionally decomposition is much faster.[16] It is assumed that the primary mode of decomposition of **2** both neat and in solution involves a free-radical chain mechanism, and consistent with this notion, the free-radical inhibitor 5-*tert*-butyl-4-hydroxy-2-methylphenyl sulfide (BHMS)[17] is found to prolong the lifetime of **2** in solution, although even in the presence of BHMS decomposition of **2** occurs. For this reason, all studies described herein were conducted with freshly prepared samples of **2** and were repeated several times to ensure reproducibility of results.

Thiol addition experiments were initially conducted with **2** and a large excess of methyl thioglycolate (0.5 M) at 23 °C, in the absence of DNA, in tetrahydrofuran (THF) containing 1,4-cyclohexadiene (1.0 M) as a hydrogen atom donor. These

(6) (a) Beerman, T. A.; Goldberg, I. H. *Biochem. Biophys. Res. Commun.* **1974**, *59*, 1254. (b) Kappen, L. S.; Napier, M. A.; Goldberg, I. H. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 1970. See also: (c) Ono, Y.; Watanabe, Y.; Ishida, N. *Biochim. Biophys. Acta* **1966**, *119*, 46. (d) D'Andrea, A. D.; Haseltine, W. A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3608.

(7) (a) Kappen, L. S.; Goldberg, I. H. *Biochemistry* **1983**, *22*, 4872. (b) Kappen, L. S.; Goldberg, I. H. *Nucleic Acids Res.* **1985**, *13*, 1637. (c) Goldberg, I. H. *Acc. Chem. Res.* **1991**, *24*, 191.

(8) Myers, A. G. *Tetrahedron Lett.* **1987**, *28*, 4493. See also ref 4c,d.

(9) (a) Chin, D.-H.; Zheng, C.-h.; Costello, C. E.; Goldberg, I. H. *Biochemistry* **1988**, *27*, 8106. (b) Chin, D.-H.; Goldberg, I. H. *J. Am. Chem. Soc.* **1993**, *115*, 9341. (c) Myers, A. G.; Cohen, S. B.; Kwon, B.-M. *J. Am. Chem. Soc.* **1994**, *116*, 1670.

(10) (a) Hatayama, T.; Goldberg, I. H.; Takeshita, M.; Grollman, A. P.; *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3603. (b) Takeshita, M.; Kappen, L. S.; Grollman, A. P.; Eisenberg, M.; Goldberg, I. H. *Biochemistry* **1981**, *20*, 7599. (c) Lee, S. H.; Goldberg, I. H. *Biochemistry* **1989**, *28*, 1019.

(11) (a) Povirk, L. F.; Houlgrave, C. W.; Han, Y.-H. *J. Biol. Chem.* **1988**, *263*, 19263. (b) Kappen, L. S.; Chen, C.-Q.; Goldberg, I. H. *Biochemistry* **1988**, *27*, 4331. (c) Frank, B. L.; Worth, L., Jr.; Christner, D. F.; Kozarich, J. W.; Stubbe, J.; Kappen, L. S.; Goldberg, I. H. *J. Am. Chem. Soc.* **1991**, *113*, 2271.

(12) (a) Povirk, L. F.; Dattagupta, N.; Warf, B. C.; Goldberg, I. H. *Biochemistry* **1981**, *20*, 4007. (b) Dasgupta, D.; Goldberg, I. H. *Biochemistry* **1985**, *24*, 6913. (c) Meschwitz, S. M.; Goldberg, I. H. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 3047. (d) Meschwitz, S. M.; Schultz, R. G.; Ashley, G. W.; Goldberg, I. H. *Biochemistry* **1992**, *31*, 9117.

(13) (a) Galat, A.; Goldberg, I. H. *Nucleic Acids Res.* **1990**, *18*, 2093. For other models of NCS chromophore binding to DNA, see: (b) Suguiyama, H.; Fujiwara, T.; Kawabata, H.; Yoda, N.; Hirayama, N.; Saito, I. *J. Am. Chem. Soc.* **1992**, *114*, 5573. (c) Elbaum, D.; Schreiber, S. L. *Bioorg. Med. Chem.* **1994**, *4*, 309.

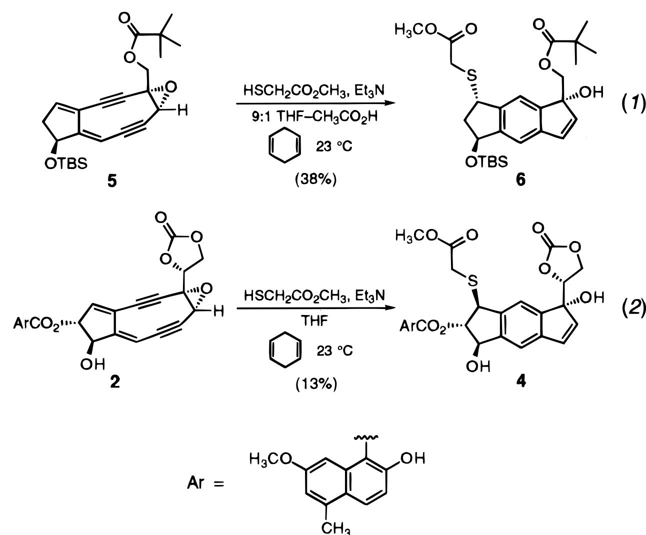
(14) Gao, X.; Stassinopolis, A.; Rice, J. S.; Goldberg, I. H. *Biochemistry* **1995**, *34*, 40.

(15) Myers, A. G.; Hammond, M.; Wu, Y.; Xiang, J.-N.; Harrington, P. M.; Kuo, E. Y. *J. Am. Chem. Soc.* **1996**, *118*, 10006.

(16) No characterizable products are evident upon analysis of the decomposition mixture.

(17) Kishi, Y.; Aratani, M.; Tanino, H.; Fukuyama, T.; Goto, T.; Inoue, S.; Sugiura, S.; Kakoi, H. *J. Chem. Soc., Chem. Commun.* **1972**, 64.

conditions were nearly identical to those of an earlier benchmark experiment that produced the neocarzinostatin chromophore−methyl thioglycolate adduct **3** (Scheme 1),[9c] but differed by the omission of acetic acid from the reaction mixture.[18] Incubation of **2** and methyl thioglycolate (3 h), as described, followed by concentration, dissolution of the residue in CD_3CN, and ^1H NMR analysis, showed that little, if any, reaction had occurred. This finding was surprising because the act of concentrating solutions of **2** is usually sufficient to induce its decomposition; more importantly, however, this result established that the aglycon (**2**) was largely inert toward methyl thioglycolate at 23 °C in the organic solvent THF. This stands in marked contrast to the behavior of the parent chromophore (**1**), which reacts with methyl thioglycolate even at −70 °C to form the cumulene intermediate (**C**),[9c] but essentially parallels earlier findings with the epoxy bicyclononadienediyne substrate **5** (eq 1).[19] Earlier experiments had established a requirement for an external base in thiol addition experiments with the latter substrate (but not **1**). Consistent with those findings, addition of triethylamine (0.5 M) to the reaction medium described containing **2** and methyl thioglycolate rapidly (≤5 min, 23 °C) consumed **2** with formation of the thiol adduct **4** as the major product (13% yield, rp-HPLC purification, eq 2).[20] Structure **4** is supported by ^1H



NMR and mass spectral data, these exhibiting considerable homology with spectra from adducts **3** and **6** obtained earlier. On the basis of $^1H-^1H$ coupling constants, the stereochemistry of the adduct **4** is assigned as trans (thioether and naphthoate groups) as it is within the adduct **3**. The formation of **4** is rationalized by the same reaction sequence previously proposed for the activation of **1** by methyl thioglycolate (Scheme 1), differing only in the nature of the the base (B:), proposed to be triethylamine in the present case and the carbohydrate amino group in the case of **1**. This proposed participation may account for the fact that thiol addition to **1** is more efficient than **2** (for conditions described, 50−70% yield versus 13% yield, respectively) and may also contribute to the faster rate and greater efficiency of DNA cleavage by **1** versus **2**, as demonstrated below.

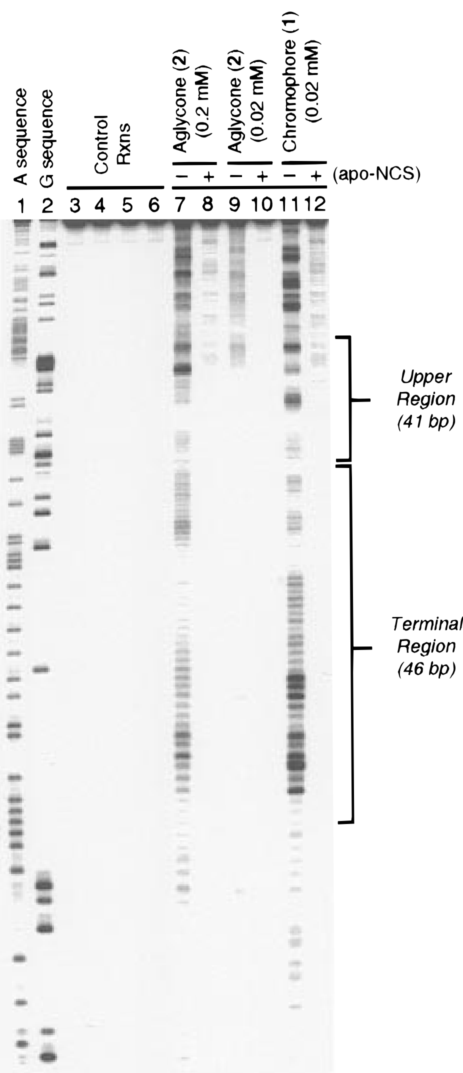


Figure 1. Thiol-dependent DNA cleavage (2 °C, 30 min) by NCS chromophore (**1**) and NCS aglycon (**2**): storage phosphor autoradiogram of an 8% denaturing polyacrylamide gel. All reaction mixtures contained calf thymus DNA (1 mM bp), 3′-labeled 193-bp restriction fragment (∼50 kcpm), and NaCl (20 mM) in tris−HCl (50 mM, pH 7.6) with 5% methanol by volume. Lanes 1 and 2: Maxam−Gilbert A and G sequencing reactions, respectively. Lanes 3 and 4: **2** (0.2 mM) and **1** (0.02 mM), respectively, no added thiol. Lanes 5 and 6: apo-NCS (0.036 and 0.36 mM, lanes 5 and 6, respectively) and methyl thioglycolate (MTG, 2 mM), no added drug. Lanes 7−12: **1** or **2** (at the indicated concentration) and MTG (2 mM) in the absence (−) or presence (+) of 1.8 equiv (with respect to the drug) of purified apo-NCS.

DNA Cleavage by NCS Aglycon (2). To evaluate the efficacy of the aglycon (**2**) in the cleavage of double-stranded DNA, parallel incubations of a 3′-^{32}P-labeled 193-base-pair (bp) restriction fragment (*Eco*RI/*Ssp*I) from plasmid pBR322 were conducted with **1** (0.02 mM) and, separately, **2** (0.02 and 0.2 mM) in the presence of methyl thioglycolate (2 mM) and calf thymus DNA (1 mM bp) at 2 °C (pH 7.6). In addition, each reaction was conducted in the presence and absence of apo-NCS (1.8 equiv with respect to the drug) to evaluate its role in the cleavage reactions. Quantitative analysis of the DNA cleavage products was achieved by polyacrylamide gel electrophoresis (PAGE) using a denaturing 8% polyacrylamide gel (Figure 1). Inspection of the data of Figure 1 shows that the

(18) Acetic acid (10% by volume) was found to stabilize the chromophore (**1**) and was present in thiol addition experiments with **1**, but promoted the rapid decomposition of **2** in solution and was therefore omitted from reactions with this substrate. The basis for this reactivity difference could not be established conclusively, for no characterizable products were obtained from the reaction of **2** with acetic acid. This finding provides another illustration of the greater chemical sensitivity of **2** as compared to **1**.

(19) Myers, A. G.; Harrington, P. M.; Kwon, B.-M. *J. Am. Chem. Soc.* **1992**, *114*, 1086.

(20) The yield reported for **4** (13%) is based on the assumption that the extinction coefficient is the same for **2** and **4** at 240 nm.

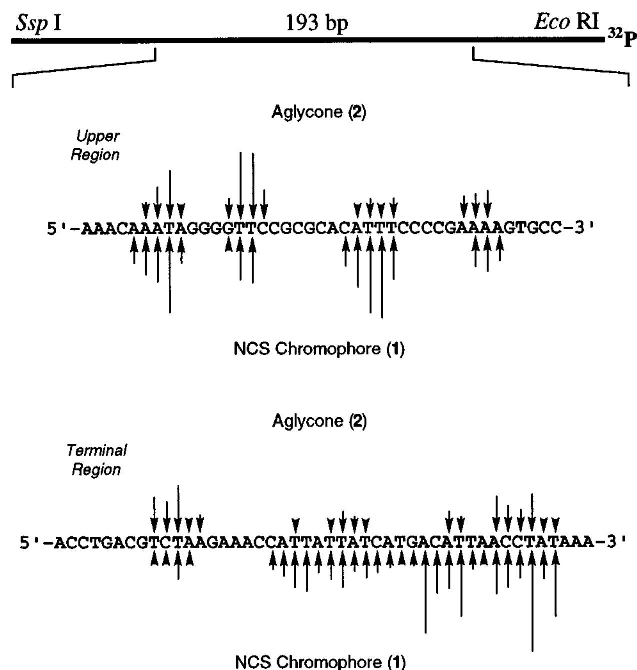


Figure 2. Observed patterns of single-stranded DNA cleavage arising from the treatment of **1** or **2** with MTG at 2 °C as described for Figure 1. Consecutive 41-bp ("upper") and 46-bp ("terminal") regions within the 3′-end-labeled 193-bp restriction fragment are identified within Figure 1. Arrow lengths are proportional to the amount of cleavage of the indicated base.

aglycon (**2**) does indeed produce single-stranded cleavage of DNA in the presence of methyl thioglycolate (but not in its absence), albeit with much lower efficiency than **1**. To achieve comparable efficiency of DNA cleavage by **2**, it was necessary to use ~10-fold higher concentrations of drug than in cleavage reactions with **1**. The cleavage efficiency for each substrate may be calculated by dividing the ratio [radioactivity of all migrating fragments]/[total radioactivity] by the ratio of drug molecules per restriction fragment. In this way, the cleavage efficiency of **1** is calculated to be 12%, versus 0.3% for **2**, for an efficiency ratio of 36 to 1.[21,22]

Like neocarzinostatin chromophore (**1**), the aglycon (**2**) is found to cleave DNA with little sequence specificity (Figure 1). Surprisingly, however, **1** and **2** show considerable similarity in their base specificites. This is perhaps most easily visualized by comparison of the histograms of Figure 2, obtained by quantitative phosphorimaging of the gel in Figure 1. The base specificities of **1** and **2** at 2 °C are calculated, respectively, as T (63%) $\gg$ A (24%) > C (11%) $\gg$ G (2%), and T (65%) $\gg$ A (22%) > C (10%) $\gg$ G (3%).[10a] Both **1** and **2** can be seen to cleave primarily at T and A residues, and largely at the same

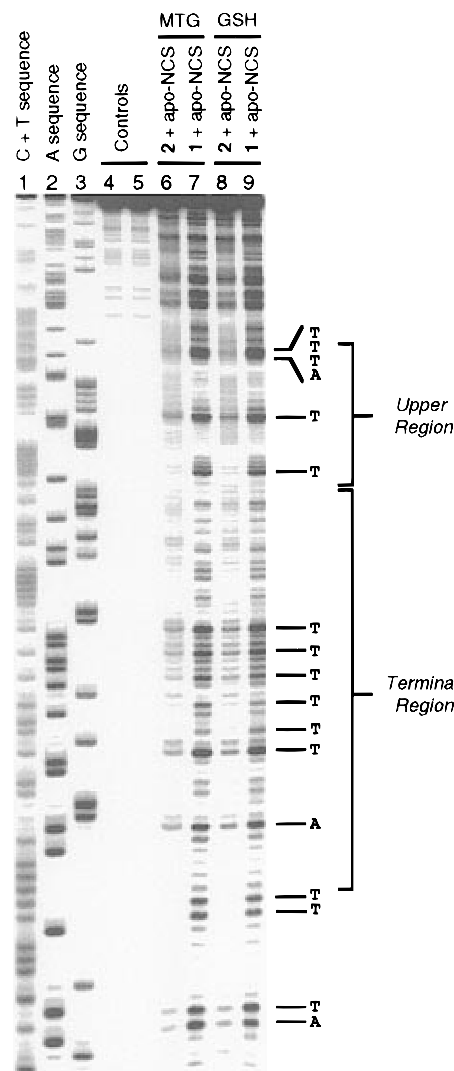


Figure 3. Comparison of thiol-dependent DNA cleavage by **1** and **2** at 37 °C using MTG (2 mM; 8 h) or GSH (2 mM; 24 h) in the presence of 1.8 equiv of apo-NCS: storage phosphor autoradiogram of an 8% denaturing polyacrylamide gel. All reaction mixtures contained calf thymus DNA (1 mM bp), 5′-labeled 193-bp restriction fragment (~50 kcpm), and NaCl (20 mM) in tris−HCl (50 mM, pH 7.6) with 5% methanol by volume. Lanes 1−3: Maxam−Gilbert C + T, A, and G sequencing reactions, respectively. Lane 4: **2** (0.2 mM) and apo-NCS (0.36 mM), no added thiol. Lane 5: **1** (0.02 mM) and apo-NCS (0.036 mM), no added thiol. Lanes 6 and 8: **2** (0.2 mM) and apo-NCS (0.36 mM) plus the indicated thiol (2 mM). Lanes 7 and 9: **1** (0.02 mM) and apo-NCS (0.036 mM) plus the indicated thiol (2 mM).

sites within the restriction fragment, although the relative intensities at specific sites do vary. This variation is found to be less pronounced in cleavage reactions conducted at 37 °C. DNA cleavage reactions at 37 °C were conducted with **1** and **2** in the presence of 1.8 equiv of apo-NCS and employed either methyl thioglycolate (MTG) or glutathione (GSH) as the activating thiol. Separate experiments were conducted with both 5′- and 3′-^{32}P-end-labeled restriction fragments to evaluate both strands for DNA cleavage and were analyzed by PAGE (Figures 3 and 4). The gel data shows a significant increase in the extent of DNA cleavage at 37 °C by **2** in the presence of apo-NCS (cleavage efficiency 0.6%, 1.8 equiv of apo-NCS, MTG activation), supporting the proposal that **2**, like **1**, is bound tightly and reversibly by apo-NCS.

By plotting the gel data of Figures 3 and 4 in histogram format (MTG activation, Figures 5 and 6) one can see that the similarity in the cleavage patterns produced by **1** and **2** (in the presence

(21) For comparison, the same 193-bp restriction fragment is cleaved with 5.7% efficiency by dynemicin A in the presence of NADPH (ref 2a) and a 35-bp restriction fragment is cleaved with ~90% efficiency by calicheamicin γ_1 in the presence of GSH (Myers, A. G.; Cohen, S. B.; Kwon, B.-M. *J. Am. Chem. Soc.* **1994**, *116*, 1255).

(22) In addition to our findings concerning the single-stranded cleavage of DNA by **1** and **2**, we investigated double-stranded cleavage within the same 5′-^{32}P-labeled 193-base pair restriction fragment using a nondenaturing electrophoretic assay (data not shown). Consistent with the generally attenuated cleavage efficiency observed for **2** relative to **1**, only traces of double-stranded cleavage by **2** (in the presence of apo-NCS) were detected. The efficiency of double-stranded DNA cleavage by **1** is at least 2−3 orders of magnitude greater than that of **2**. These findings also held true in the case of piperidine treatment (1 M piperidine, 90 °C, 30 min) of the cleavage products: (a) Poon, R.; Beerman, T. A.; Goldberg, I. H. *Biochemistry* **1977**, *16*, 486. (b) Dedon, P. C.; Goldberg, I. H. *J. Biol. Chem.* **1990**, *265*, 14713. (c) Kappen, L. S.; Goldberg, I. H. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6706.

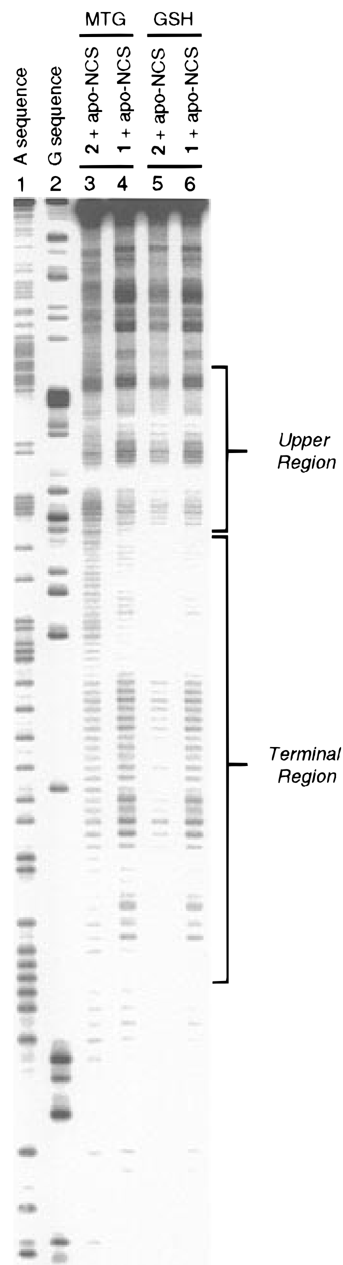


Figure 4. Comparison of thiol-dependent DNA cleavage by **1** and **2** at 37 °C using MTG (2 mM; 8 h) or GSH (2 mM; 24 h) in the presence of 1.8 equiv of apo-NCS: storage phosphor autoradiogram of an 8% denaturing polyacrylamide gel. All reaction mixtures contained calf thymus DNA (1 mM bp), 3′-labeled 193-bp restriction fragment (~50 kcpm), and NaCl (20 mM) in tris−HCl (50 mM, pH 7.6) with 5% methanol by volume. Lanes 1 and 2: Maxam−Gilbert A and G sequencing reactions, respectively. Lanes 3 and 5: **2** (0.2 mM) and apo-NCS (0.36 mM) plus the indicated thiol (2 mM). Lanes 4 and 6: **1** (0.02 mM) and apo-NCS (0.036 mM) plus the indicated thiol (2 mM).

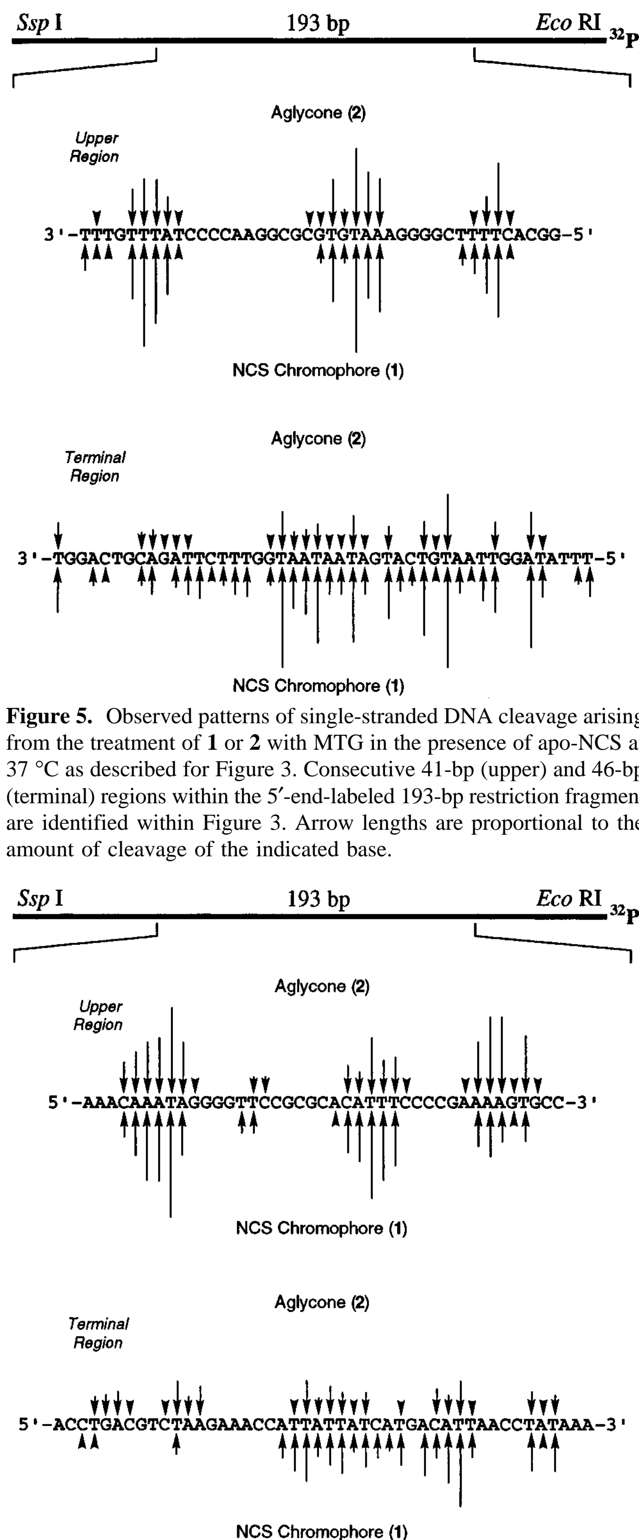


Figure 5. Observed patterns of single-stranded DNA cleavage arising from the treatment of **1** or **2** with MTG in the presence of apo-NCS at 37 °C as described for Figure 3. Consecutive 41-bp (upper) and 46-bp (terminal) regions within the 5′-end-labeled 193-bp restriction fragment are identified within Figure 3. Arrow lengths are proportional to the amount of cleavage of the indicated base.

Figure 6. Observed patterns of single-stranded DNA cleavage arising from the treatment of **1** or **2** with MTG in the presence of apo-NCS at 37 °C as described for Figure 4. Consecutive 41-bp (upper) and 46-bp (terminal) regions within the 3′-end-labeled 193-bp restriction fragment are identified within Figure 4. Arrow lengths are proportional to the amount of cleavage of the indicated base.

of apo-NCS) is even greater at 37 °C than at 2 °C. Quantitative analysis of the base specificities of DNA cleavage by **1** and **2** for both methyl thioglycolate- and glutathione-induced cleavage reactions at 37 °C in the presence of apo-NCS shows a good correspondence: for the 3′ strand, **1** and **2**, respectively, T (64%) ≫ A (27%) > C (8%) ≫ G (1%), and T (57%) ≫ A (32%) > C (8%) ≫ G (3%); for the 5′ strand, **1** and **2**, respectively, T (70%) ≫ A (22%) > C (6%) ≫ G (2%), and T (66%) ≫ A (21%) > C (11%) ≫ G (2%). Goldberg and co-workers[10b] have previously shown that the DNA cleavage pattern produced by **1** is unaffected by the presence of apo-NCS and, consistent with this finding, control experiments (DNA cleavage with **1** and **2** at 37 °C in the absence of apo-NCS, data not shown) reveal that the variations in the patterns of Figures 2 and 6 are attributable to temperature effects and not the presence of apo-NCS. These temperature effects in cleavage selectivity are significant and underscore the kinetic nature of the cleavage reactions by these highly reactive species.

Comparative Kinetics of DNA Cleavage by NCS Chro-

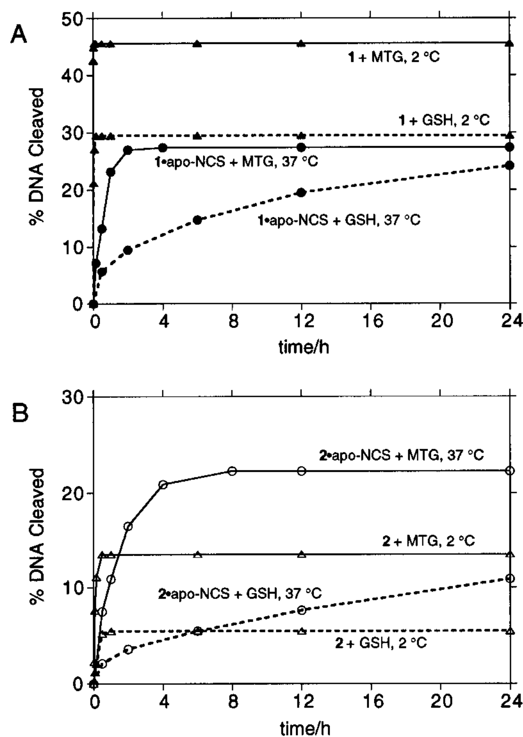


Figure 7. Kinetics of thiol-dependent DNA cleavage by (**A**) NCS chromophore (**1**, 0.02 mM) and (**B**) aglycon (**2**, 0.2 mM) using MTG (2 mM) or GSH (2 mM) in the presence or absence of apo-NCS (1.8 equiv). Reactions were performed in tris−HCl (50 mM, pH 7.6) containing calf thymus DNA (1 mM bp), 3′-labeled 193-bp restriction fragment (∼150 kcpm), and NaCl (20 mM) with 5% methanol by volume: (▲) **1**, 2 °C; (△) **2**, 2 °C; (●) **1**, apo-NCS, 37 °C; (○) **2**, apo-NCS, 37 °C; (—) reactions containing MTG; (- - -) reactions containing GSH.

mophore and NCS Aglycon (2). To assess the rates of DNA cleavage by **1** and **2**, kinetic profiles were obtained for both methyl thioglycolate- and glutathione-induced activation, in both the presence and absence of apo-NCS, and are illustrated in Figure 7. Analysis of the kinetics data shows that the reactions of **1** with MTG and GSH are both faster than the corresponding reactions with the aglycon (**2**). The following rate constants were obtained for DNA cleavage under the pseudo-first-order conditions of excess thiol (2 mM) and in the absence of apo-NCS: **1** and MTG, 17 M^{-1} s^{-1} ($t_{1/2} \sim 20$ s); **1** and GSH, 4.1 M^{-1} s^{-1} ($t_{1/2} \sim 84$ s); **2** and MTG, 1.5 M^{-1} s^{-1} ($t_{1/2} \sim 230$ s); **2** and GSH, 0.83 M^{-1} s^{-1} ($t_{1/2} \sim 420$ s). Thus, the rate of DNA cleavage by **1** in the presence of MTG is 11-fold faster than **2**, while GSH activation leads to a 5-fold rate difference in DNA cleavage.[23]

Discussion

Role of the Carbohydrate Residue in Chromophore Reactivity. The rate of spontaneous decomposition of NCS aglycon (**2**) neat or in solution is much greater than for the glycosylated natural product (**1**). In this regard, the carbohydrate residue clearly provides a stabilizing influence upon the chromophore core. We suspect that the primary mode of decomposition of **1** and **2** in an inert solvent or in neat form involves a free-radical chain pathway, perhaps by addition of a free radical to the strained cyclononadiyne ring followed by transannular radical cyclization and chain propagation. In support of this proposal is the fact that free-radical inhibitors markedly extend the lifetimes of **1** and **2** neat or in solution. The stabilizing role of the carbohydrate residue is believed to be steric in nature, slowing the approach of free radicals to the chromophore core.

Although the carbohydrate residue may slow the rate of spontaneous decomposition of **1** in solution, a large body of evidence has now been accrued that demonstrates that the carbohydrate amino group plays a major role in accelerating thiol activation of **1**. Earlier model studies revealed a requirement for an external base in thiol addition to C-12 in chromophore models lacking the amino sugar component (e.g., substrate **5**). Similar findings with NCS aglycon (**2**) described above provide the most compelling evidence to date in support of the function of the amino sugar as an internal base in the thiol activation of **1**. The effect is dramatic; addition of methyl thioglycolate to **1** has been shown to occur readily at −70 °C, whereas its addition to the aglycon (**2**) is slow or does not occur at 23 °C. The proposed participation of the aminoglycoside residue as an internal base in thiol addition to C-12 is also supported by X-ray crystallography of the neocarzinostatin protein−chromophore complex, wherein the carbohydrate amino group is found to lie 5 Å above C-12, a distance approximately equal to the van der Waals diameter of a sulfur atom.[5]

We have previously shown that the rate-determining step in DNA cleavage by **1** and methyl thioglycolate in water (in the absence of apo-NCS) is the thiol addition reaction and, further, that this thiol addition reaction occurs as a ternary complex of **1**, thiol, and DNA at the concentrations of these experiments.[9c] It is tempting to speculate that the slower rate of DNA cleavage by **2** reflects an intrinsically slower rate of thiol addition to **2** versus **1**, perhaps due to an accelerating effect by the carbohydrate amino group of **1**, but this cannot be stated with certainty. It may also be, for example, that **2** binds to DNA in such a fashion that C-12 is less accessible to nucleophilic thiol than in **1** bound to DNA. The variance in rates is almost certainly associated with the thiol addition step, however, for once cumulene formation occurs the subsequent events of Scheme 1 are all believed to be quite rapid.

DNA cleavage reactions with NCS chromophore (**1**) using GSH as the activating thiol show similar or greater cleavage efficiency in the presence of apo-NCS than without, but reactions with MTG show the opposite trend. That is to say, MTG-induced cleavage reactions with **1** are less efficient in the presence of apo-NCS than in its absence. This observation has already been noted by Saito and co-workers[24] and is readily understood, as shown by those investigators, by the fact that small thiols such as MTG undergo a competitive protein-directed reaction with **1** (a reaction manifold not available to larger thiols such as glutathione), thereby reducing the overall efficiency of the cleavage reaction. We recently established the structure of this protein-directed thiol adduct and proposed a mechanism for its formation.[25] It is significant that the aglycon (**2**) does not show a similar reduction in cleavage efficiency with MTG (Figure 7), which we interpret to mean that **2** bound to apo-NCS does not undergo a corresponding protein-directed side reaction. We suggest that this may again reflect the absence of an internal base in **2**, now in reference to protein-directed thiol addition chemistry. The implication of this proposal is that the aminoglycoside of **1** may function as an internal base both in the protein-directed addition reaction of **1** with small thiols and in the normal course of thiol activation of **1** bound to DNA.

(23) The rate of activation of **1** by GSH is known to be slower than with MTG: Dedon, P. C.; Goldberg, I. H. *Biochemistry* **1992**, *31*, 1909.

(24) (a) Sugiyama, H.; Yamashita, K.; Nishi, M.; Saito, I. *Tetrahedron Lett.* **1992**, *33*, 515. (b) Sugiyama, H.; Yamashita, K.; Fujiwara, T.; Saito, I. *Tetrahedron* **1994**, *50*, 1311.

(25) Myers, A. G.; Arvedson, S. P.; Lee, R. W. *J. Am. Chem. Soc.* **1996**, *118*, 4725.

Evidence for the Binding and Stablization of NCS Aglycon (2) by apo-NCS. The data of Figure 1 show that the presence of the neocarzinostatin binding protein (apo-NCS, 1.8 equiv per drug molecule) effectively abolishes DNA cleavage by both **1** and **2** at 2 °C (30 min reaction time). This result was anticipated for **1**,[26] where it has been established that the presence of apo-NCS dramatically slows the DNA cleavage reaction by its tight binding of the chromophore (K_D of the protein−chromphore complex of $\sim 10^{-10}$ M versus K_D of $\sim 10^{-6}$ M for **1** bound to double-stranded DNA),[27] but could not be predicted with certainty for **2**. Mass spectral studies of **2** had earlier provided the first indication that the aglycon (**2**) was capable of binding to apo-NCS.[15,28] Although the data of Figure 1 support the idea that the aglycon (**2**) binds tightly to apo-NCS, thereby retarding the rate of DNA cleavage by **2** in analogy to **1**, an alternative interpretation is that apo-NCS induces the decomposition of **2**. This interpretation is discounted by the observation that DNA cleavage by **2** in the presence of apo-NCS is markedly increased at 37 °C and with extended reaction times (Figure 3).[29,30] These findings provide compelling evidence that NCS aglycon (**2**), like NCS chromophore (**1**), is bound tightly and reversibly by apo-NCS and that this binding stablizes the aglycon in solution.

Role of the Carbohydrate Residue in the Sequence Specific Cleavage of DNA. Perhaps the most surprising result from DNA cleavage experiments described herein is the correspondence in base specificities observed for **1** and **2**, suggesting that the aminoglycoside plays little role in determining the base specificity of DNA cleavage by neocarzinostatin chromophore (**1**). It is also evident from inspection of the gel data of Figures 1, 3, and 4 (and has been previously shown for **1**[31]) that the base specificities of **1** and **2** are essentially unaffected by the nature of the activating thiol. The data suggest that substituents residing trans to the naphthoate residue (thioether and aminosugar) do not markedly influence the specificity of DNA cleavage by **1**. As previously noted, the species that determines the specificity of DNA cleavage by **1** is likely not the biradical intermediate **B** (Scheme 1), but rather, the cumulene intermediate **C**, although the two intermediates may be quite similar in their binding to DNA.[32] We propose that the primary recognition element for the base specific cleavage of DNA by **1** and **2** is composed of the planar naphthoate residue and the orthogonally disposed π-face of the cyclic cumulene cis to the napthoate residue. These two perpendicular π-surfaces may provide a shape-selective recognition element for DNA binding.[33] From an analysis of space-filling molecular models, we propose that the transition state for hydrogen atom abstraction from the 5′ position of a thymidine undergoing attack by C-6 of the biradical **B** involves a more deeply penetrated activated chromophore than a simple intercalation model would suggest, such that van der Waals contacts between the indacene π-face that is cis to the naphthoate residue and the thymidine ribose ring are established. Such a proposal is also consistent with the somewhat unusual 5′-hydrogen atom abstraction by **1**, for the 5′-hydrogens of DNA are not readily accessible to a minor groove-binding molecule without substantial deformation of the B-form DNA helix.

Findings from several laboratories have established that, in contrast to **1**, the carbohydrate domain is largely responsible for the sequence specificity of DNA cleavage by the aminoglycoside antibiotic calicheamicin γ_1.[34] It should be noted that the carbohydrate domain of calicheamicin γ_1 is much larger than that of **1** and that calicheamicin γ_1 also exhibits greater sequence specificity in DNA cleavage than **1**.[34d] Within the enediyne antibiotics, the aminoglycoside residues of calicheamicin γ_1 and, as described herein, neocarzinostatin appear to play a functional role, improving the rate and efficiency of DNA cleavage. By contrast, Hecht et al.[35] and Umezawa et al.[36] have found that deglycobleomycin−Fe(II) is only slightly less active as a DNA cleaving agent than the parent antibiotic, bleomycin−Fe(II), and produces an almost identical pattern of DNA cleavage. Taken together, these findings suggest that no common paradigms have yet emerged for the role of the carbohydrate residues of the enediyne antibiotics, or of free-radical-based DNA-modifying natural products in general.

Experimental Section

Neocarzinostatin protein−chromophore complex was generously provided by Kayaku Co., Ltd., and was stored as a dry powder at −80 °C. NCS chromophore (**1**) was extracted from neocarzinostatin (holo-NCS) following published procedures.[9c] The preparation of the 3′- and 5′-^{32}P-end-labeled 193-base-pair restriction fragments has been previously described.[2b,9c] Methanolic solutions of aglycon (**2**) were prepared as previously described[15] and were used immediately, being stored briefly at −80 °C when necessary. All manipulations of **1** and **2** were conducted at 4 °C and in the absence of light whenever possible. Extinction coefficients for **1** and apo-NCS were determined experimentally: neocarzinostatin chromophore (**1**), λ (ϵ, M^{-1} cm^{-1}) (MeOH)

(26) (a) Kappen, L. S.; Napier, M. A.; Goldberg, I. H. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 1970. (b) Koide, Y.; Ito, A.; Ishii, F.; Koyama, Y.; Edo, K.; Ishida, N. *J. Antibiot.* **1982**, *35*, 766.

(27) Free chromophore binds to poly(dA-dT) with a rate constant of 7.8 × 10^5 M^{-1} s^{-1} at 2 °C. Dissociation of the chromophore from the bound complex occurs more slowly, with a rate of 21 s^{-1}, corresponding to a half-life of ∼0.03 s. (a) Povirk, L. F.; Goldberg, I. H. *Biochemistry* **1980**, *19*, 4773. (b) Dasgupta, D.; Auld, D. S.; Goldberg, I. H. *Biochemistry* **1985**, *24*, 7049.

(28) Electrospray mass spectroscopy of aqueous solutions of **2** containing excess apo-NCS (18 equiv) was found to produce ions consistent with a 1:1 complex of **2** and apo-NCS while all attempts to obtain a mass spectrum of **2** in isolation were unsuccessful.

(29) Goldberg and co-workers have previously shown that holo-NCS (**1**·apo-NCS) efficiently cleaves double-stranded DNA at 37 °C (ref 7c).

(30) Kinetic monitoring of DNA cleavage reactions conducted at 2 °C with **1** and **2** in the presence of apo-NCS (1.8 equiv) showed a slow but real increase in DNA cleavage as a function of time.

(31) The nature of the thiol cofactor is known to influence the extent of bistranded DNA lesions (ref 11b) and the partitioning of the chemistry of deoxyribose damage: (a) Saito, I.; Kawabata, H.; Fujiwara, T.; Sugiyama, H.; Matsuura, T. *J. Am. Chem. Soc.* **1989**, *111*, 8302. (b) Dedon, P. C.; Jiang, Z.-W.; Goldberg, I. H. *Biochemistry* **1992**, *31*, 1917 and references cited therein. (c) Kappen, L. S.; Goldberg, I. H.; Frank, B. L.; Worth, L., Jr.; Christner, D. F.; Kozarich, J. W.; Stubbe, J. *Biochemistry* **1991**, *30*, 2034.

(32) This proposal was previously set forth (ref 9c) on the basis of an analysis of the estimated rate constants for hydrogen atom abstraction by the biradical intermediate and on/off rates for the binding of such an intermediate to DNA (ref 27). Even considering the important recent findings of Chen et al., in which the rate of hydrogen atom abstraction by a biradical such as **B** is revised downward, the rate of such a hydrogen atom abstraction is still estimated to be faster than equilibration of the biradical with another site of intercalation: (a) Logan, C. F.; Chen, P. *J. Am. Chem. Soc.* **1996**, *118*, 2113. (b) Schottelius, M. J.; Chen, P. *J. Am. Chem. Soc.* **1996**, *118*, 4896.

(33) (a) Barton, J. K. *Science* **1986**, *233*, 727. (b) Pyle, A. M.; Mori, T.; Barton, J. K. *J. Am. Chem. Soc.* **1990**, *112*, 9432. (c) Sitlani, A.; Long, E. C.; Pyle, A. M.; Barton, J. K. *J. Am. Chem. Soc.* **1992**, *114*, 2303. See also: (d) Dickerson, R. E.; Drew, H. R. *J. Mol. Biol.* **1981**, *149*, 761.

(34) (a) Zein, N.; Poncin, M.; Nilakantan, R.; Ellestad, G. A. *Science* **1989**, *244*, 697. (b) Drak, J.; Iwasawa, N.; Danishefsky, S. J.; Crothers, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7464. (c) Walker, S.; Landovitz, R.; Ding, W.-D.; Ellestad, G. A.; Kahne, D. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4608. (d) Aiyar, J.; Hitchcock, S. A.; Denhart, D.; Liu, K. K. C.; Danishefsky, S. J.; Crothers, D. M. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 855. (e) Paloma, L. G.; Smith, J. A.; Chazin, W. J.; Nicolaou, K. C. *J. Am. Chem. Soc.* **1994**, *116*, 3697. See also: (f) Kahne, D. *Chem. Biol.* **1995**, *2*, 7.

(35) (a) Oppenheimer, N. J.; Chang, C.; Chang, L.-H.; Ehrenfeld, G.; Rodriguez, L. O.; Hecht, S. M. *J. Biol. Chem.* **1982**, *257*, 1606. (b) Hamamichi, N.; Natrajan, A.; Hecht, S. M. *J. Am. Chem. Soc.* **1992**, *114*, 6278.

(36) (a) Sugiura, Y.; Suzuki, T.; Otsuka, M.; Kobayashi, S.; Ohno, M.; Takita, T.; Umezawa, H. *J. Biol. Chem.* **1983**, *258*, 1328. (b) Umezawa, H.; Takita, T.; Sugiura, Y.; Otsuka, M.; Kobayashi, S.; Ohno, M. *Tetrahedron* **1984**, *40*, 501.

302 nm (7040), (0.5 M AcOH/MeOH) 302 nm (5830); apo-NCS, λ (ϵ, M^{-1} cm^{-1}) (H_2O) 340 (1060), 302 (2030), 277 (9300), 270 nm (8840). The extinction coefficients determined for NCS chromophore (**1**) were also used for the aglycon (**2**).

Preparation of Methyl Thioglycolate Adduct 4. 2,5-Dimethoxybenzyl alcohol (ca. 0.6 mg) was added as an internal standard to a deoxygenated solution of the aglycon **2** in methanol (0.5 mL of a ~2 mg/mL solution) containing BHMS (ca. 0.05 mg), and the ratio of aglycon/internal standard was determined by rp-HPLC analysis (ODS C18 column, 10 mm × 25 cm, isocratic solvent system of 40% 10 mM ammonium acetate (pH 5.5)/60% acetonitrile, flow rate of 2.0 mL/min, monitoring at 240 nm; retention times: **2**, 25.0 min; **4**, 14.1 min; 2,5-dimethoxybenzyl alcohol, 7.3 min). The solution was concentrated to a volume of ca. 0.1 mL, the concentrate was diluted with toluene (0.5 mL), and the resulting solution was concentrated to a volume of ca. 0.1 mL. This procedure was repeated twice. Tetrahydrofuran (0.2 mL) and 1,4-cyclohexadiene (0.30 mmol, 24 mg, 0.028 mL, final concentration 1.0 M) were added to the concentrate, and the solution was deoxygenated. A deoxygenated solution of methyl thioglycolate (0.15 mmol, 16 mg, 0.013 mL, final concentration 0.5 M) and triethylamine (0.15 mmol, 15 mg, 0.021 mL, final concentration 0.5 M) in tetrahydrofuran (0.1 mL) was added, and after 55 min, the volatiles were removed in vacuo. The residue was dissolved in toluene, and the resulting solution was concentrated to ca. 0.1 mL. This procedure was repeated twice. The residue was then taken up in acetonitrile (0.3 mL), and the yield of **4** was determined by rp-HPLC analysis (as above, 13%). This procedure was repeated on a 2-fold larger scale, and the product was isolated by rp-HPLC (as above) to afford the adduct **4** as a pale yellow oil (ca. 0.2 mg): ^{1}H NMR (400 MHz, CD_3CN) δ 8.10 (d, J = 9.53 Hz, 1 H; C4″ H), 7.78 (br s, 1 H; C8″ H), 7.57 (s, 1 H; C2 H), 7.35 (s, 1 H; C8 H), 7.04 (d, J = 9.16 Hz, 1 H; C3″ H), 6.92 (d, J = 5.88 Hz, 1 H; C6 H), 6.84 (br s, 1 H; C6″ H), 6.26 (d, J = 5.84 Hz, 1 H; C5 H), 5.75 (t, J = 3.32 Hz, 1 H; C11 H), 5.34 (br m, 1 H; C10 H), 4.67 (dd, J = 8.44, 5.52 Hz, 1 H; C13 H), 4.60 (d, J = 3.28 Hz, 1 H; C12 H), 4.43 (t, J = 9.16 Hz, 1 H; C14 H), 4.27 (dd, J = 8.76, 5.84 Hz, 1H; C14 H), 3.59 (s, 3 H; C7″ $OC\mathbf{H}_3$), 3.54 (abq, J = 15.36 Hz, ν = 46.97 Hz, 2 H; $SC\mathbf{H}_2$), 3.48 (s, 3 H; $CO_2C\mathbf{H}_3$), 2.57 (s, 3 H; C5″ $C\mathbf{H}_3$); FTIR (CH_3CN) 3628 (s, OH), 3542 (s, OH), 2932 (m), 1732 (m, C=O), 1631 (m, C=O), 1278 (m), 1204 (m), 1009 cm^{-1}(w); MS (electrospray) m/z 631 [M^+ + Na], 647 [M^+ + K]; TLC (10% CH_3OH/CH_2Cl_2) R_f 0.42 (fluoresces under UV, anisaldehyde).

DNA Cleavage Experiments with 1 and 2. Reactions of **1** and **2** with GSH or MTG were performed in 1.6 mL Eppendorf tubes in a total reaction volume of 200 μL. A 20 μL aliquot of an aqueous solution of double-stranded calf thymus DNA (10 mM base pair) was combined with 3′-^{32}P-labeled 193-base-pair restriction fragment (5 × 10^5 cpm), an aqueous solution of GSH or MTG (20 μL, 20 mM, pH 7.5), NaCl (8 μL, 500 mM), and tris−HCl aqueous buffer solution (20 μL, 500 mM, pH 8.1). For reaction mixtures containing **1**, 5 μL of methanol was added, and for reaction mixtures containing aglycon **2**, 5 μL of 0.5 M AcOH/methanol was added, thereby affording a final pH of 7.6 in both solutions following the addition of drug. For reactions that incorporated apo-NCS, an aqueous solution of purified apo-NCS (1.0 mM)[37] was added such that 1.8 equiv of apo-NCS was present in each reaction mixture with respect to **1** or **2**. Sufficient water was added to each reaction solution prior to addition of drug to achieve a final volume of 200 μL. Reactions were incubated at 2 or 37 °C for 15 min prior to the addition of drug. Reactions were initiated by the addition of a cold (−78 °C) solution of either **1** (5 μL, 0.8 mM) in 0.5 M AcOH/methanol or **2** (5 μL, 8.0 or 0.8 mM) in methanol, producing the following concentrations of solution components at the onset of the reaction: **1** (0.02 mM) or **2** (0.2 or 0.02 mM); double-stranded calf thymus DNA (1.0 mM base pair); apo-NCS (0.36 or 0.036 mM); GSH or MTG (2.0 mM); NaCl (20 mM); tris−HCl buffer (50 mM). Control reactions were carried out on a 1/4 scale (50 μL total volume) in identical fashion with the omission of the appropriate reaction component (drug, thiol, apo-NCS). All reactions were incubated at the indicated temperatures in the absence of light. For kinetics experiments, 35 μL aliquots of a given reaction solution were withdrawn at the indicated time intervals and were added to an aqueous solution of sodium acetate (5 μL, pH 5.2) and ethanol (150 μL), and the products were analyzed subsequently by gel electrophoresis, as previously described.[2,9c]

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(37) Denklan, D.; Köhnlein, W.; Lüders, G.; Stellmach, J. *Z. Naturforsch.* **1983**, *38c*, 939.