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DNA cleavage characteristics of non-protein enediyne antibiotic N1999A2☆

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Abstract

N1999A2 (NA2) is a new non-protein antitumor antibiotic that contains a stable 9-membered ring enediyne chromophore similar to a neocarzinostatin chromophore (NCS-chr). We have compared DNA cleavage reactions between NA2 and NCS-chr, and also clarified some characteristics of DNA strand scission by NA2. It was found that: (1) NA2 is considerably stable in nature, (2) the compound intercalates into base pairs of a DNA minor groove and decreases its base-attacking frequency in the order of T>A>> C>G, (3) the base-sequence specificity 5′-GGT/3′-CCA presented by NA2 is significantly related to recognition of the base pair with the naphthoate moiety, and (4) the different cleavage property between NCS-chr and NA2 is associated with the presence or absence of an aminoglycoside residue. Based on the results of the site-specific cleavage by NA2 for certain bulged DNAs and a fluorescence study of NA2–DNA oligomer complexes, the DNA interaction mode of NA2 has also been examined. These results provide important information to design a new enediyne molecule for a DNA target.

The new antibiotic N1999A2 (NA2), isolated from a culture supernatant of *Streptomyces* sp. AJ9493 [1], possesses a naphthoate moiety and a novel 9-membered ring enediyne chromophore (Fig. 1). NA2 strongly inhibited the growth of tumor cell lines and bacteria. The IC_{50} values of cytotoxicity for various cell lines in vivo were 10^{-12}–10^{-8} M. Neocarzinostatin chromophore (NCS-chr) [2], C-1027 [3], esperamycin A^1 [4], and calichemicin γ_1 [5] belong to the enediyne-containing antitumor antibiotic family and also cleave a DNA strand through hydrogen abstraction from the deoxyribose of DNA.

The chemical structure of NA2 is remarkably similar to that of NCS-chr, except that the latter contains an aminoglycoside residue. The activation of NCS-chr depends on thiol agents such as dithiothreitol (DTT), methyl thioglycolate, and glutathione [6]. NA2 is sufficiently stable during the isolation procedure in spite of the non-protein chromophores. This point is of special interest and different from the other 9-membered ring enediyne chromophore. In the case of NCS, its apo-protein non-covalently binds to the labile chromophore and contributes to the great stabilization of the NCS-chr [7,8]. NA2 is more stable than NCS-chr in aqueous solution. Although NA2 can cleave DNA even in the absence of thiol agents, the presence of thiol agents accelerates the activation of NA2 further. DNA cleavage, its inhibition, and fluorescence experiments revealed the cleavage property and sequence specificity in the double-strand DNA by NA2. The investigation of bulged DNA clarified that the aminoglycoside moiety of NCS-chr plays a dominant role in specific cleavage at the T_{22} site [9,10]. The present results indicate DNA cleavage characteristics by the novel enediyne compound NA2. In addition, the role of the aminoglycoside in NCS-chr has been discussed on the basis of the comparative results between NA2 and NCS-chr.

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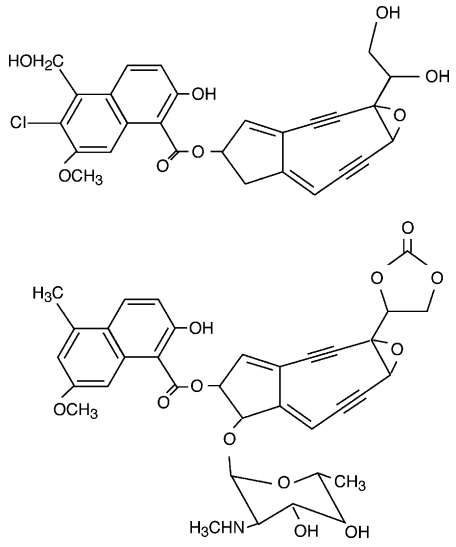

Fig. 1. Chemical structures of NA2 (upper) and NCS-chr (lower).

Materials and methods

Drugs and chemicals. NA2 was obtained according to the previous report [1], and NCS-chr was a gift from Kakenyaku Co. Distamycin A was offered by Dr. F. Arcamone (Farmitalia). These antibiotics were stored at −80 °C, protected against light, and dissolved immediately in refined methanol before use. Oligonucleotides were purchased from Amersham Pharmacia Biotech and plasmid pBR322 DNA was isolated from *Escherichia coli* C600. Bacterial alkaline phosphatase, *E. coli* DNA polymerase I large fragment, T4 polynucleotide kinase, and restriction enzymes were purchased from Takara Shuzo (Kyoto, Japan). All other chemicals were of commercial reagent grade.

Labeling of oligonucleotides. Some oligonucleotides were labeled at the 5′-terminus with [γ-^{32}P]ATP using T4 polynucleotide kinase. After purification on a denaturing 15% polyacrylamide gel, each oligonucleotide was allowed to anneal with the opposite strand.

Dependence of cleavage activity on pH and temperature. Using acetate and Tris buffers, the pH value was varied from 4.0 to 9.0. Each sample contained 1 μM pBR322 plasmid DNA. The reaction was initiated by addition of 200 μM NA2 and 300 μM DTT, and carried out for 20 min. On the other hand, the reaction temperature was altered from 4 to 45 °C. The reaction samples contained 1 μg pBR322 DNA and 20 mM Tris–HCl buffer (pH 7.0). The reaction was started with 160 μM NA2 and 300 μM DTT, and carried out for 20 min. Electrophoresis of these samples was carried out using 1% agarose gel containing ethidium bromide (0.5 μg/ml).

Stability and DNA cleavage of NA2 and NCS-chr. NA2 (160 μM) or NCS-chr (80 μM) was preincubated with 20 mM Tris–HCl buffer (pH 7.0) for 15 min at 37, 50, and 70 °C, and then pBR322 DNA (1 μg) and DTT (100–300 μM) were added to the samples. Electrophoresis of these samples was performed with 1% agarose containing ethidium bromide (0.5 μg/ml). The reaction mixture (total volume, 20 μl) contained the 5′-end-labeled *Sal*I–*Nru*I pBR322 DNA fragment, 1 μg of sonicated calf thymus carrier DNA, 10% (v/v) methanol, and 20 mM Tris–HCl buffer (pH 7.0). The 5′-end-labeled oligonucleotide was treated with NA2 or NCS-chr and analyzed by electrophoresis on a 10% sequencing gel.

Effect of distamycin A on DNA cleavage by NA2. The 5′-end-labeled *Sal*I–*Nru*I pBR322 DNA fragments were preincubated with distamycin A at 37 °C for 30 min. NA2 (200 μM) and DTT (300 μM) were added to the reaction samples and then the reaction was carried out at 37 °C for 20 min. The cleaving reaction was stopped by addition of ethanol and sodium acetate (0.3 M), and the DNAs were then recovered by ice-cold ethanol precipitation. The inhibition sites were analyzed by electrophoresis on a 10% denaturing sequencing gel.

Specific double-stranded DNA cleavage. Each 5′-end-labeled oligonucleotide (T/A and C/G types) was annealed with the other complementary strand in 50 mM NaCl and 50 mM Tris–HCl buffer (pH 7.0) at 80 °C. The cleavage reaction was initiated by addition of NA2 (200–600 μM) and DTT (300 μM) in 20 mM Tris–HCl buffer (pH 7.0) containing 0.1 μg calf thymus DNA. The cleaved base sites were detected using electrophoresis on a 15% denaturing polyacrylamide gel at 2500 V for 1 h.

Fluorescence studies. In the DNA intercalation experiment, fluorescence measurements were performed using a Hitachi F-3010 spectrofluorometer at 20 °C. Extinction was at 239 nm and the emission spectrum was recorded from 350 to 470 nm. After the oligomer duplex was preincubated with 5 μM post-activated NA2 at 4 °C, fluorescence quenching was measured in the sample solution containing 300 mM sodium acetate and 1% methanol. The concentration of the oligomer duplex was varied from 2 to 10 μM. If only one binding site per DNA duplex can be assumed, the dissociation constant (K_d) was calculated from the fluorescence quenching data.

Cleavage of bulged DNA by NA2. The 5′-end-labeled oligomers (31 mer) were self-annealed in 50 mM Tris–HCl buffer (pH 7.0) containing 1 mM EDTA by heating at 90 °C for 2 min and by fast cooling to 4 °C. The reaction samples (total volume, 25 μl) contained 160 μM NA2, 50 mM Tris–HCl buffer (pH 7.0), 1 mM EDTA, the ^{32}P-end-labeled bulged DNA, and non-labeled bulged DNA. The samples were chilled at 4 °C for 10 min prior to the addition of NA2. The cleavage reactions were allowed to proceed at 4 °C for 24 h.

Results and discussion

Effect of thiol, pH, temperature, and UV on DNA cleavage by NA2

As shown in Table 1, some thiols significantly stimulate DNA breakage of NA2 from covalently supercoiled (form I) pBR322 DNA to open-circular (form II) and linear (form III) DNAs. The DNA cutting ability of NA2 decreases in the order of methyl thioglycolate > 2-mercaptoethanol = DTT > glutathione. Table 2 shows the effect of pH on the NA2-mediated DNA breakage, indicating that its DNA cleavage is strong near the neutral pH region. NA2 showed apparent DNA cleavage activity even at 4 °C and also exhibited potent DNA breakage ability in the range of 20–45 °C. As well as thiols, UV-light irradiation also significantly induced DNA strand scission by NA2. Previously,

Table 1
Effect of various reductants on DNA cleavage by N1999A2 (160 μM) at 37 °C and pH 7.0 for 20 min

Reductants (50 μM)	Form of DNA (%)		
	Form I	Form II	Form III
Blank	91.2	8.8	0.0
Mercaptoethanol	29.9	69.3	0.8
Methyl thioglycolate	0.0	87.7	12.3
Glutathione	57.2	42.8	0.0
Dithiothreitol (DTT)	29.5	70.5	0.0

Table 2
Effect of pH on DNA cleavage by N1999A2 (200 μM) at 37 °C for 20 min

Reaction pH	Form of DNA (%)		
	Form I	Form II	Form III
Blank	90.3	9.7	0.0
pH 4.0	80.7	9.6	9.7
pH 5.0	57.2	31.2	11.6
pH 6.0	16.5	36.0	47.5
pH 7.0	12.0	34.4	53.6
pH 8.0	21.8	28.4	49.8
pH 9.0	51.3	44.7	4.0

we found visible light-induced DNA cleavage by dynemicin A [11].

Stability of NA2

In order to examine the stability of NA2 in solution, we checked the DNA cleavage activity of NA2. After NA2 was preincubated with 20 mM Tris–HCl buffer (pH 7.0) at 37, 50, and 70 °C for 15 min, pBR322 DNA and DTT were added to each sample, and the sample solution was incubated at 37 °C for 20 min. The quantity of residual form I DNA is an index of the inactivation of NA2. The inactivation of NA2 was 2.1% (37 °C), 27.5% (50 °C), and 59.3% (70 °C). On the other hand, the inactivation of NCS-chr was 19.4%, 54.2%, and 97.6%, respectively, under the same condition (date not shown). Indeed, NA2 was considerably stable at 37 °C and the decomposition rate of NA2 was much slower than that of NCS-chr. It is of special interest that non-protein NA2 exists as a 9-membered ring enediyne chromophore, because other non-chromoproteins are 10-membered ring endiynes such as dynemicin A, esperamycin A_1, and calichemicin γ_1.

Comparison of NA2 and NCS-chr in DNA cleavage

Fig. 2 shows a typical autoradiographic result with the 5′-end-labeled 321-bp pBR322 DNA fragment for DNA strand scissions by NA2 and NCS-chr in the presence of DTT. The strong DNA damage by NA2 was clearly observed at thymidine and adenine base sites (Fig. 3). The frequency (T>>A>C>G) of the attacked bases is very similar to that of NCS-chr (T>A>>C>G), but is different from those of C-1027 (A ⩾ T>>C>G) [3], esperamycin A_1 (T>C>A>G) [4], and calichemicin γ_1 (C>>T>A≅G) [5]. However, a more random DNA cleavage profile of NA2 than NCS-chr was detected (Fig. 3). It has been proposed that the methylamino group of the NCS-chr aminoglycoside moiety participates in the specific recognition of the thymine base by forming a hydrogen bond with the C-2 carbonyl of the thymine base [12], and that the naphthoate ring undergoes stacking interaction with DNA bases [13,14]. Probably, the naphthoate ring of NA2 is also necessary for interaction with DNA. Thymine is the best cleaved base for NA2 similar to NCS-chr. Indeed, the preincubation of the post-activated (aromatized) NCS-chr specifically inhibited the most cleaved thymine sites by NA2 (data not shown). Consequently, the naphthoate moiety of NA2 likely prefers to intercalate into the AT base pair. In NCS-chr, the less random cleavage and more specific thymine attack may be caused by hydrogen bond formation between the aminoglycoside and thymine.

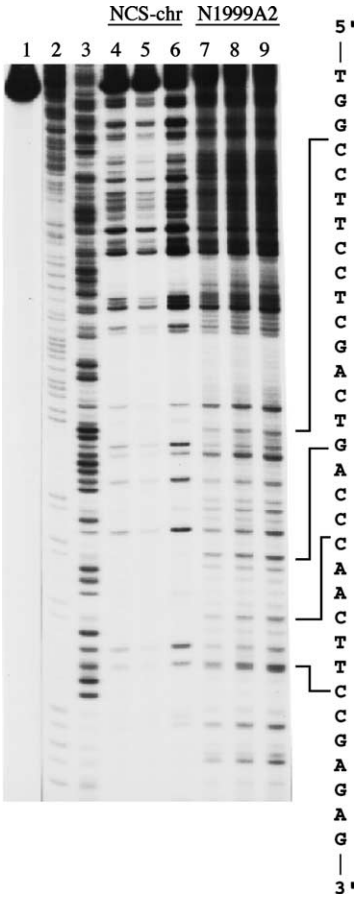

Fig. 2. Comparison of thiol-dependent DNA cleavages by NA2 and NCS-chr. The samples contain the following concentrations of NCS-chr: lane 4, 60 μM; lane 5, 80 μM; and lane 6, 100 μM, and the concentrations of NA2: lane 7, 100 μM; lane 8, 150 μM; and lane 9, 200 μM. Lanes 1, 2, and 3 indicate intact DNA alone, G + A, and C + T of the Maxam–Gilbert sequencing reactions, respectively.

Inhibition of NA2-mediated DNA cleavage by distamycin A

Distamycin A is a well-known minor groove binder for specific AT-rich sequences [15]. Fig. 4 shows the alteration of DNA strand scission induced by NA2 after pretreatment with distamycin A. The DNA cutting sites of NA2 were clearly inhibited with increasing

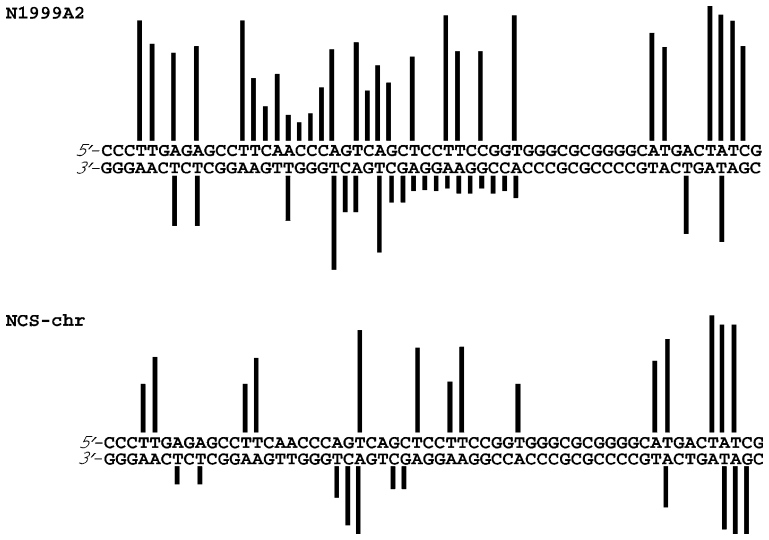

Fig. 3. Histogram of pBR322 *Sal*I–*Nru*I fragment cutting sites by NA2 or NCS-chr.

concentrations of distamycin A (Fig. 4). In particular, the significant protection sites were detected in AT-rich regions such as 5′-CTATCG, 5′-GTCAG, 5′-TCCTTCC, and 5′-AGCCTTC. This result indicates that NA2 interacts with a DNA duplex through the DNA minor groove and that its biradical enediyne abstracts hydrogen from the sugar phosphate backbone of DNA. As with NCS-chr, the naphthoate moiety of NA2 presumably intercalates with AT bases in the minor groove of DNA.

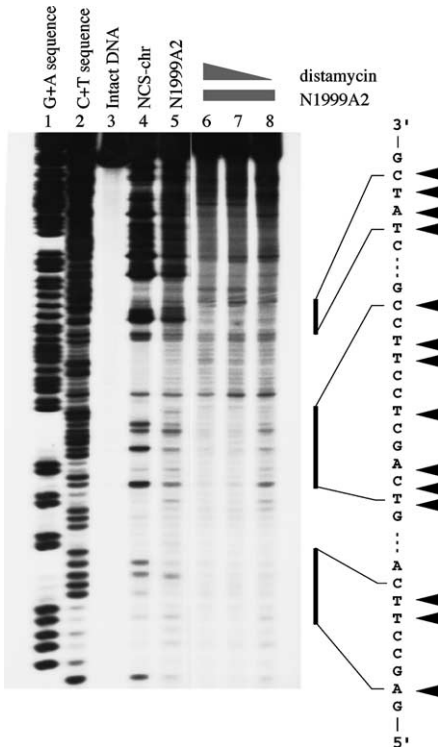

Fig. 4. Inhibition of NA2-induced DNA cleavage activation by distamycin A. After 30-min preincubation with distamycin A of 100 μM (lane 6), 60 μM (lane 7), and 20 μM (lane 8) at 37 °C, the NA2 thiol-activated reactions were carried out at 37 °C for 20 min. Lanes 4 and 5 reveal cleavage patterns by NCS-chr and NA2 in the absence of distamycin A, respectively. Lane 2 exhibits DNA sequencings (G + A and C + T, respectively) by the Maxam–Gilbert method and lane 3 shows intact DNA alone.

Double-stranded DNA cleavage

The characteristic double-stranded breakage induced by NA2 was investigated. Two types (T/A and G/C types) of DNA were incubated with thiol-activated NA2 at 37 °C for 20 min. As shown in Fig. 5, the autoradiograph showed typical double-stranded cleavage at 5′-GGT/3′-CCA for the T/A type DNA. This result resembles the NCS-chr-cleaving patterns, namely 5′-GCT/3′-CGA and 5′-AGT/3′-TCA [16]. Indeed, both the antibiotics cause double-stranded breaks two base pairs apart at specific sites. In addition, such an asymmetric cleavage pattern with the 3′-side on the opposite strand suggests interaction of NA2 within the minor groove of DNA duplex. On the other hand, no specific double-stranded cleavages by NA2 were detected in the C/G type DNA (data not shown). Using molecular dynamics calculation and NMR methods, a three-dimensional structure for a complex of the aromatized NCS-chr and duplex DNA containing the sequence of 5′-GCT/3′-CGA has been proposed [17,18]. The naphthoate moiety intercalates into the CT/AG pair, and also C6 of the aromatized NCS-chr is close to the thymine base (C5′) of one DNA strand and C2 of the NCS-chr is near the cytosine base (C1′) of the opposite strand. Therefore, the C6 and C2 biradicals of NCS-chr can abstract hydrogens from the thymidine (C5′) and cytosine (C1′), respectively [19]. On the basis of the results

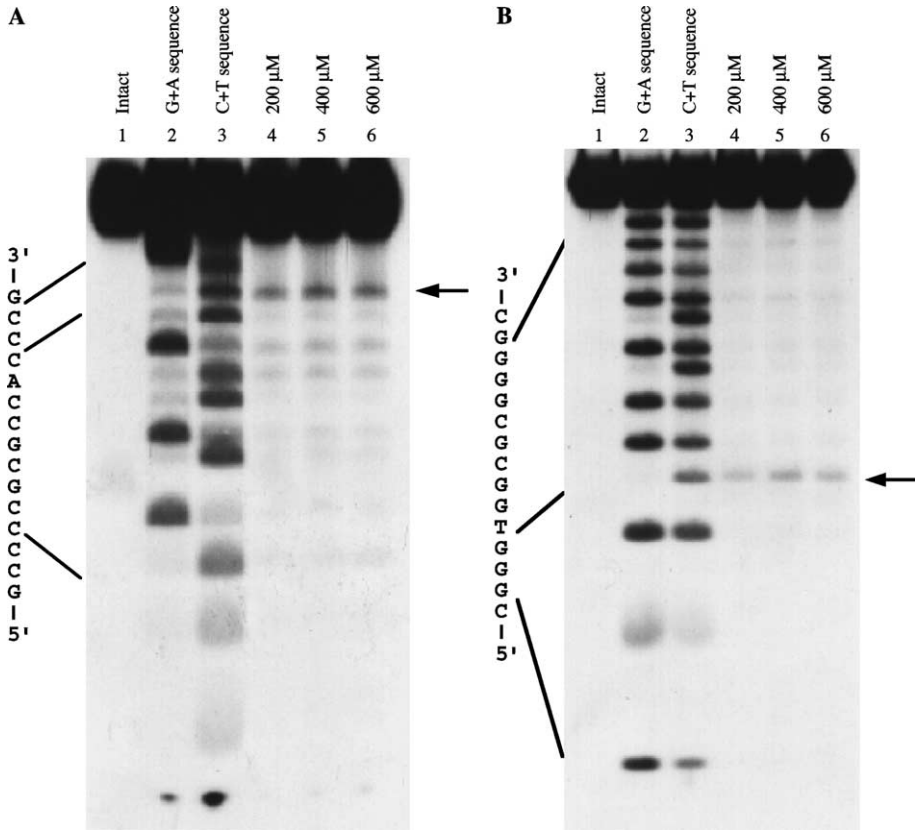

Fig. 5. Strand scission of the 5′-end-^{32}P-labeled T/A type DNA sequence by NA2. (A) The cleavage pattern of the strand containing adenine. The concentrations of NA2 were 200 μM (lane 4), 400 μM (lane 5), and 600 μM (lane 6). (B) The scission pattern of the complementary strand. The concentrations of NA2 were 200 μM (lane 4), 400 μM (lane 5), and 600 μM (lane 6). Lanes 1, 2, and 3 show DNA intact alone, DNA sequencings (G + A and C + T) by the Maxam–Gilbert method, respectively.

concerning NCS-chr, the double-stranded cleavage elicited by NA2 is assumed similarly, except for the aminosugar moiety.

Fluorescence study

Fig. 6 presents the fluorescence spectral change of NA2, depending on the concentration of T/A type DNA. Increasing amounts of T/A type DNA resulted in an increase in the fluorescence quenching of aromatized NA2, until its spectrum reached saturation. The dissociation constant (K_d) of the NA2–DNA complex and the number of binding sites (N) were calculated from the Scatchard plot. In the case of T/A type DNA, the K_d value was 6.7 μM and the N value was 1.18. In the case of C/G type DNA, however, the Scatchard plot exhibited no specific bindings, and hence the K_d value could not be estimated (data not shown). The K_d and N values reveal high specificity of NA2 for certain target steps within T/A type DNA. On the other hand, the increase in NA2 fluorescence quenching in C/G type DNA may be attributed to either non-specific interaction or the binding of self-stacked molecules of the cationic drug to polyanionic DNA [17].

Cleavage of bulged DNA by NA2

It is well known that NCS-chr cleaves a specific site of single-stranded DNA containing a bulged structure in

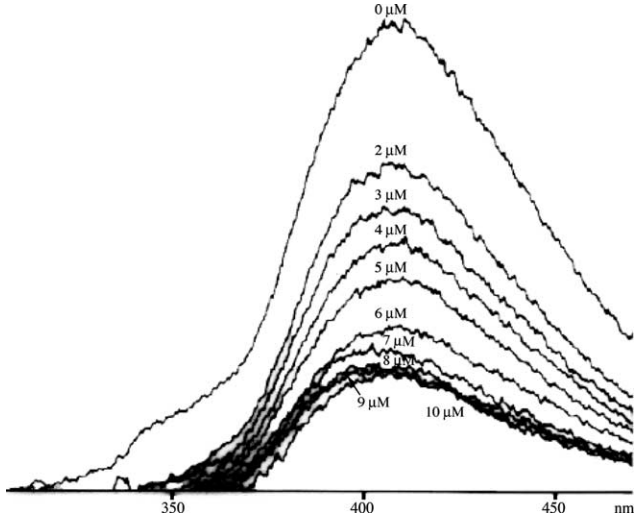

Fig. 6. Fluorescence spectra of post-activated NA2 depending on the concentrations of T/A type DNA. Each sample contains 300 mM sodium acetate (pH 5.2) buffer and 1% methanol. Excitation was at 239 nm and emission at 406 nm.

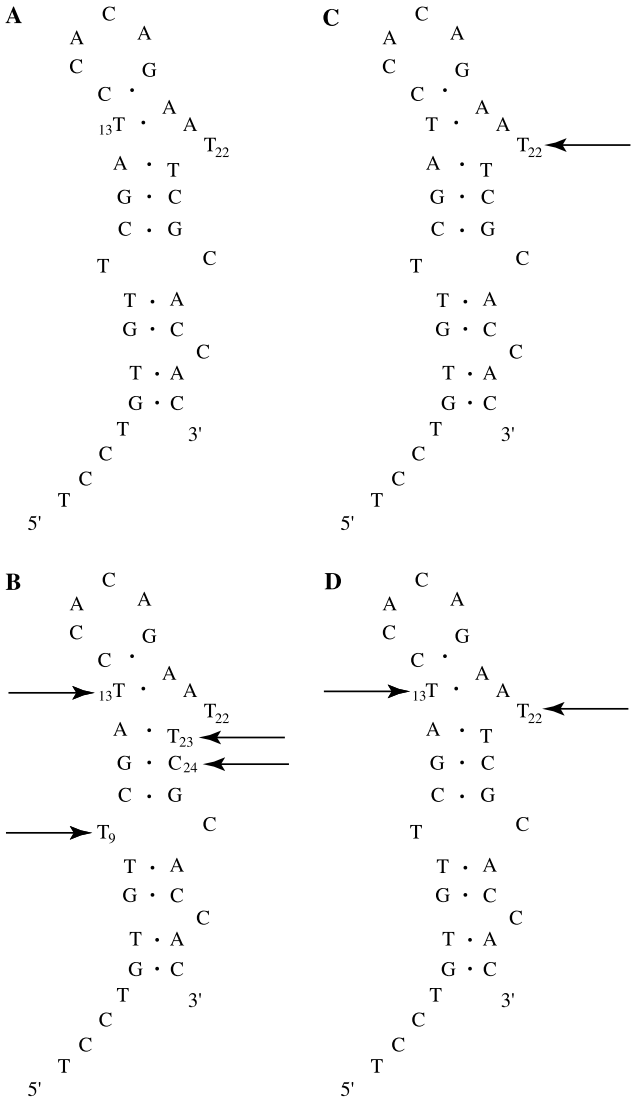

Fig. 7. Cleavage sites of bulged DNA by NA2 (A, B) and NCS-chr (C, D) in the absence (A, C) and presence (B, D) of thiol agent. Arrow shows cleavage site.

the absence of a thiol agent [9,10]. The single-stranded DNA is an analogue of the 3′-terminal fragment (31 mer) of yeast $tRNA^{phe}$ and the bulged DNA is cleaved only at the T_{22} site under aerobic condition (Fig. 7). Herein, a similar reaction of NA2 instead of NCS-chr was carried out in the absence of thiol under aerobic condition. NA2 cleaved neither at the bulge T_{22} site nor other base sites under the same experimental condition. In the presence of a thiol agent, on the other hand, cutting sites were observed at the T_9, T_{13}, T_{23}, and C_{24} base sites, but not the T_{22} site (Fig. 7). In contrast, NCS-chr cleaved T_{13} and T_{22} sites in the presence of thiol. The difference in the thiol-independent cleavage at T_{22} between NA2 and NCS-chr may be due to the absence or presence of aminoglycoside residues in the antibiotic molecules. The previous NMR study revealed that the naphthoate moiety in NCS-chr interacts with the base pair of bulged DNA through stacking interaction and that the aminoglycoside contacts the T_{22} base of bulged DNA [20]. Presumably, the aminoglycoside moiety plays an important role in site-specific binding of the antibiotic at the pocket of bulged DNA.

In conclusion, NA2 existed as a stable non-chromoprotein in spite of the 9-membered enediyne ring and induced a strong DNA cleavage reaction depending on pH in the presence of thiol. The base-specific DNA cutting by NA2 is similar to that by NCS-chr, but NA2 more randomly cleaved the double-stranded DNA. Although NCS-chr resulted in highly specific breakage at the base T_{22} site for the bulged DNA in the absence of thiol, NA2 induced no cleavages for the bulged DNA under the same experimental conditions. Presumably, this difference is due to the absence or presence of an aminoglycoside moiety in the enediyne chromophores. The present information on NA2 would be useful for the design and creation of new and effective enediyne compounds.

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