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Probing DNA Bulges with Designed Helical Spirocyclic Molecules

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Abstract

Since bulged structures (unpaired bases) in nucleic acids are of general biological significance, it has been of interest to design small molecules as specific probes of bulge function. Based on our earlier work with the specific DNA bulge-binding metabolite obtained from the enediyne antitumor antibiotic neocarzinostatin chromophore (NCS-chrom) we have prepared three small helical spirocyclic molecules that most closely mimic the natural product. These wedge-shaped molecules resemble the natural product in having the sugar residue attached to the same 5-membered ring system. In one instance the sugar is aminoglucose in β-glycosidic linkage and in the other, two enantiomers having the natural sugar N-methylfucosamine in α-glycosidic linkage. All three analogues were found to interfere with bulge-specific cleavage by NCS-chrom and with the ability of bulged-DNA to serve as a template for DNA polymerase 1 in accord with their binding affinities for DNA containing a 2-base bulge. Comparable results were obtained with the analogues for the less efficiently cleaved 3-base bulge DNA structures. In each situation the enantiomers possessing the natural sugar in α-glycosidic linkage are the most potent inhibitors of the cleavage reaction. In the DNA polymerase reactions again the closest natural product mimics were the most effective in selectively impeding nucleotide extension at the bulge site, presumably by complex formation. These results demonstrate the potential usefulness of bulge-binding compounds in modifying DNA structure and function and support efforts to design and prepare reactive species of these molecules that can covalently modify bulged DNA.

Bulged structures in nucleic acids are of general biological significance because of the multitude of roles ascribed to them in a number of biochemical processes (1,2). They have been proposed as intermediates in RNA splicing, frame-shift and intercalator-induced mutagenesis, binding motifs for regulatory proteins in viral replication as in the case of the TAR region of HIV and as essential elements in naturally occuring antisense RNAs (3). Bulged structures have also been implicated as intermediates in slipped DNA synthesis associated with the unstable expansion of triplet repeats in several neurodegenerative diseases, such as Huntington's disease, Friederich's ataxia, and fragile X syndrome, as well as in nucleotide expansions found in certain human cancers (4-7). Thus, considering the importance of bulge structures in a variety of biological processes, small molecules capable of selectively targeting nucleic acid bulges will be valuable probes to study their role(s) in nucleic acids function.

Our earlier work shows that the enediyne antitumor antibiotic neocarzinostatin (NCS-chrom), upon general base-catalyzed intramolecular activation via a biradical species (Scheme 1), cleaves DNA selectively at bulge sites, preferably of 2−3 unpaired bases (8-10). The major

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metabolite generated in the base-catalyzed reaction in the absence of DNA is a wedge-shaped spirolactone molecule (Scheme 1, **1**) that closely resembles the biradical and shows high binding affinity to DNA bulges with great specificity (11). NMR analysis of its complex with bulge DNA showed that the binding to the bulge involves major groove recognition by its amino sugar moiety and tight fitting of the wedge-shaped helical molecule in the triangular prism pocket formed by the two looped-out DNA bulge bases and the neighboring base pairs (12-13), with which the drug ring systems stack. Furthermore, this agent was also found to induce the formation of a bulge binding pocket in an oligonucleotide of relatively unstructured form and to interact with it (14). The high lability of the spirolactone and its limited availability however, precluded its use in detailed biological studies. We, therefore, have been interested in synthesizing stable small molecules, designed after the natural metabolite that are capable of preferential binding to DNA bulge sites. The synthetic analogues, while possessing a stable spirocyclic core with a right-handed twist of about 35° as in the natural product, differed from it in certain features including the nature of the sugar moiety and its linkage. They all showed DNA bulge-specific binding of varied affinities, as monitored by distinctive fluorescence changes upon binding, but were significantly weaker than the natural metabolite (15-17). The analogues also stimulated DNA slippage synthesis occurring at nucleotide repeats in in vitro expansion systems containing a series of primers/templates and the Klenow fragment of *E. coli* DNA polymerase 1 (18), presumably by binding to or inducing the formation of bulge structures, the postulated intermediates in DNA strand slippage, resulting in enhanced synthesis.

In order to obtain compounds with high affinity for nucleic acid bulges, three compounds modeled more closely after the bulge-binding metabolite **1** of NCS-chrom by having the sugar moiety attached to the same 5-membered ring system as in the natural product (Figure 1) were synthesized (16 and 19). All three closely mimic **1** in their spirocyclic core structure but differ from it in their sugar moiety and/or its linkage. In **2** the sugar is glucosamine with a β− glycoside linkage ; enantiomers **3** and **4** having N-methylfucosamine in an α-glycosidic bond closely resemble **1**. Binding studies, based on fluorescence changes, showed that **3** and **4** (K_d= ~0.1 μM) have stronger affinity than **2** (K_d= ~0.6 μM) for DNA bulges (19). All three compounds also stimulated DNA slippage synthesis in assays using simple repeat sequences and DNA polymerase 1 (16,19).

Despite the evidence for bulge-specific binding by the analogues, their usefulness depends on how the binding affects subsequent biological events, and this would largely be governed by the architecture and local geometry of the drug-DNA complexes. In the present study we sought to establish the biological significance of bulge binding of the analogues first by assessing their ability to block bulge-specific strand cleavage by NCS-chrom, the most potent and highly specific strand cleaver at DNA bulges. Secondly, it would be of interest to know if the bulge-binders affect DNA synthesis on a DNA template having a putative bulge. The instability of the natural metabolite **1** precluded its use in such experiments. With stable analogues now on hand, such studies are possible. The results obtained in these two approaches shed light on the biological significance of their bulge binding with regard to DNA structure and function and show the potential use of synthetic analogues in studies involving nucleic acid bulges.

MATERIALS AND METHODS

Materials

The following materials were purchased from the sources indicated: Oligodeoxyribonucleotides (primers and templates), Integrated DNA Technologies or Midland Certified Company; deoxynucleoside triphosphates, Amersham Pharmacia; Radioactive materials, New England Nuclear; T4 polynucleotide kinase, Klenow fragment of *E. coli* DNA polymerase 1, New England Biolabs. The primers were 5'-^{32}P-end-labeled using [γ-^{32}P] ATP and polynucleotide kinase. The labeled oligomers were purified by electrophoresis on a 15 % denaturing gel by standard procedures (20). The products, eluted from the gel slices were purified using a desalting Sephadex column (Pharmacia).

DNA Cleavage and Competition Assays

NCS-chromophore was extracted from the holoantibiotic by cold methanol containing 0.5 M acetic acid by a procedure similar to that described by Myers (21). Bulge-containing DNA duplexes (Table 1) were prepared by annealing in Tris-HCl buffer pH 8.0, two oligomers, the longer of which carried ^{32}P label at its 5'-end . Generally, the short strand was in 2−3 fold excess. The annealed DNA was treated with NCS-chrom as previously reported (9). A standard reaction (30−40 μl) on ice contained 50 mM Tris-HCl pH 8.0, 5−10 μM annealed DNA duplex and the drug at levels indicated in the Figure Legends . In competition experiments DNA was preincubated with varying levels of the test compound for 10−15 minutes on ice prior to the addition of NCS-chrom. The reaction was terminated by the addtion of NaOH to a final level of 50 mM followed by neutralization with an eqivalent amount of HCl. Raising the pH with NaOH inactivates NCS-chrom instantly. Portions of the reaction mixtures were dried and the pellets were dissolved in 80% formamide, containing marker dyes. After separation of the products on a 15% sequencing gel, the band intensities were quantitated on a Phophor Imager (Molecular Dynamics).

Markers to identify the DNA fragments from the drug reaction were prepared by chemical cleavage (G+A and T+C) of the 5'-^{32}P-end-labeled oligomers according to Maxam and Gilbert method (20).

DNA Polymerase Assays

The primers/ templates used in this study are listed in Table 1. A mixture of the 5'-^{32}P-end-labeled primer(1 μM) and unlabeled template, generally the latter in 3 fold excess, was annealed by heating in Tris-HCl pH 7.5, to 95° followed by slow cooling to room temperature. The concentrations of the components at the annealing stage were 30−50% higher than those in the final assay to accommodate the dilution resulting from the addition of the rest of the components in the subsequent stage. While the reaction conditions varied slightly in different experiments, a standard reaction (30 μl) contained 50 mM Tris-HCl, pH 7.5, 1 μM annealed duplex (based on the primer), 5 mM $MgCl_2$, 3 mM dithiothreitol, 0.8 mM each of the deoxynucleoside triphosphates and the Klenow fragment of DNA polymerase I at different levels. After the addition of the test compounds (as a solution in 50% dimethyl sulfoxide) the mixture was preincubated at room temperature for 10 min. Controls lacking the drug received an equal volume of 50% dimethyl sulfoxide; the final concentration of which was 2% in the assays. The reaction was started by the addition of the enzyme. The incubation was at 33^0 for times indicated in the Figure Legends. The reaction was terminated by the addition of EDTA to a final concentration of 40 mM. Portions of the reaction mixture were dried, and analyzed on a 15% sequencing gel. The gels were exposed to X-ray film and the band intensities were quantitated on a Phosphor Imager.

RESULTS and DISCUSSION

Effect of DNA Bulge-Binding Analogues on Strand Cleavage at a 2-Base Bulge by NCS-chrom

Previous studies have shown that NCS-chrom induces efficient strand cleavage exclusively at DNA bulges in a general base-catalyzed reaction via a mechanism involving a biradical (Scheme 1) (8). As illustrated in Scheme 2 with a bulge-containing DNA duplex (Table 1, entry 1), NCS-chrom attack at the 5' position of the target T_8 (arrow) results in two fragments: (a) 5'-^{32}P-end-labeled fragment having phosphate at its 3' end and (b) an unlabeled oligomer with a nucleoside aldehyde at its 5' end. A compound that is capable of binding to the bulge site is likely to block cleavage if it has the same binding mode, precise geometry and microstructure in the bulge cavity as the cleaving agent, as was the case with the natural metabolite **1** (11).

In experiments shown in Figure 2, DNA duplex 1 of Table 1 that was prepared by annealing a 12-mer and 5'-^{32}P-end-labeled 14-mer, and containing a two-base bulge was treated with NCS-chrom (15 μM) in the absence and presence of **4**. Analysis of the products on a sequencing gel shows that the drug induced site-specific cleavage at T_8 of the bulge resulting in a single ^{32}P band of the cleaved product (lanes, 2 and 3, arrow) depicted as fragment (a) in Scheme 2 . Addition of **4** inhibited cleavage in a dose-dependent manner (lanes 4–8). A plot of the inhibition versus the concentration of **4** gave fifty percent inhibition at 62 μM of **4**. These results are in accord with the relative bulge-binding efficiencies of the NCS-chrom metabolite **1** and **4** as assessed in fluorescence-based assays where the latter (K_d = 0.1) was found to have weaker bulge-binding affinity than **1** (K_d = 0.03). The ability of **4** to inhibit strand cleavage suggests that its complex at the bulge site is similar, if not identical, to that of **1**, in its binding mode and microstructrure. A time course of the reaction using the bulge duplex 2 of Table 1 (Figure 3) shows that strand scission by NCS-chrom reaches a plateau at about 40 min, and inhibition by **4** remains somewhat steady over the entire period.

Comparison of the Three Analogues in the Inhibition of Strand Cleavage

In experiments similar to those in Figure 3, analogues **2**, **3** and **4** were compared for their ability to inhibit strand cleavage by NCS-chrom. After separation of the products by gel analysis, the band intensities were quantitated. Figure 4 shows that **3** and **4** having N-methylfucosamine in α- glycosidic linkage efficiently inhibited cleavage. Fifty percent inhibition was obtained at a level of these compounds three to four-fold in excess of NCS-chrom (**3**, 50 μM; **4**, 85 μM). On the other hand, **2** which has an aminoglucose moiety in β- glycosidic linkage, is the least efficient of the three, and it caused only 35 % inhibition at the highest level (150 μM) used. These results show that the sugar structure and the nature of the glycosidic bond in the bulge-binder are important determinants of its binding affinity, stability and conformation of its complex at the bulge site.

Inhibition of Strand Cleavage at 3-Base Bulges by 4

Three base bulges are functionally important motifs in nucleic acids, especially in RNAs such as HIV-1 TAR RNA. Previous studies showed that NCS-chrom cleaved at 3-base bulges in DNA and HIV-1 TAR RNA though much less efficiently than at 2-base bulges (22). Unlike in the case of 2-base bulges, strand scission occurred at more than one base in the bulge with varying intensities. Experiments shown in Figure 5 were performed using duplexes 3 and 4 (Table 1) having ^{32}P label at the 5' ends of their bulge strands. Both duplexes are identical except for the sequence difference in the 3-base bulge. Treatment of duplex 3 (lanes 1–7), which has the bulge sequence 5'-GCT with NCS-chrom generates two bands ((lanes 4 and 5 and arrows). The strong band is the expected cleavage product of attack at T_9, and the minor band with mobility slightly faster than that of the main product results from attack at C_8 with cleavage of 13% and 5%, respectively. Both were inhibited by **4** to the same extent (72%). There is a distinct difference in the cleavage pattern obtained with duplex 4 having 5'-GTC bulge sequence (lanes 8–14) where cleavage (26%) is almost exclusive at C_9 (lanes 9 and 10) and is also much stronger than that at T_9 of the GCT sequence. C_9 cleavage is inhibited 57% in the presence of **4**. The efficiency of total cleavage at the GTC bulge (34%) is nearly twice as much as that obtained with the GCT bulge. Thus, despite the sequence preference of NCS-chrom, **4** inhibited the strand cleavage in both the bulge substrates (lanes 6, 7 and 11, 12). The finding that **4** is able to inhibit strand cleavage in 2-base (Figure 2) and 3-base bulges (Figure 5) shows that its complex at the bulge has a conformation and microstructure very similar to that with the cleaving agent, NCS-chrom, which has proved to be a versatile molecule that can inflict a variety of lesions in DNA depending upon its mode of activation, the sequence and the structure of the DNA and accessibility of the drug to the various attack positions in the deoxyribose of the target nucleotide (13).

In addition to bulge-specific cleavage, NCS-chrom also induces strand breaks in duplex DNAs mainly at T and A residues in the presence of a thiol activator by a mechanism involving thiol adduction and its rearrangement via a cumulene intermediate to a non-spirocyclic diradical species (13). A Watson-Crick duplex (Table 1, duplex 5) was prepared by annealing 5'-^{32}P-end-labeled 10-mer with its complementary 10-mer and treated with NCS-chrom in the presence of a thiol. Addition of **4** to this reaction did not significantly affect the extent of cleavage (data not shown).

Effect of Synthetic Analogues on DNA Polymerase Reactions

Small molecular probes that can selectively bind to nucleic acid bulges at specific locations so as to influence DNA/RNA synthesis will be valuable tools for studies in biological systems. It was hence of interest to test whether the binding of the synthetic analogues at DNA bulge sites would affect DNA synthesis. This would require a DNA template having a two-base bulge at a defined position and also a single-stranded tail at its 3'end to complement with a primer that can be extended in a polymerase-dependent reaction. We have previously used a single-stranded 31-mer (Table 1, entry 9) that has the potential to fold to generate a stable hairpin structure with a loop and a two-base bulge as in the putative folding pattern shown in Figure 6A (8). NCS-chrom induced very efficient strand cleavage exclusively at its 2-base bulge in base-catalyzed, oxygen-dependent reactions (8,9), whereas under anaerobic reactions it formed a stable covalent adduct at the bulge instead of a strand break. When the isolated adduct-bound 31-mer was used as a template in DNA polymerase-dependent primer extension reactions, the synthesis was blocked at the adduct site (23).

Although the bulge-binding of the synthetic analogues is noncovalent, we used the same strategy in the design of experiments to determine their effect on DNA synthesis. The 31-mer was annealed with a 5'-^{32}P-end-labeled primer (Table 1, entry 6–8) that is complementary, starting from its 3'-end overhang, with the assumption that in the annealed duplex the folded structure with the bulge (Figure 6A) would be maintained. DNA polymerase-dependent extension of the primer (Figure 6C) was then followed in the absence and presence of **4**. A gel profile of the reaction products at three time points is shown in Figure 7. In the controls lacking the test compound the synthesis produced full length products as well as a series of weak bands which are revealed only on an overexposed film (lanes 3, 4, 5 and 9, 10, 11). In the presence of **4** (also **3**, data not shown)) there is an enhancement of band intensities selectively at positions 21–23 (arrows) which comprise the bulge site (lanes 6, 7, 8). In experiments (not shown) the band lengths at the bulge region were confirmed by running in parallel lanes 5'-^{32}P labeled oligomers of 20–25 nucleotides in length and sequence of the expected products with 3' hydroxyl groups. Quantitation of the bands at 21–23 positions (Table 2) shows that the enhancement of their intensities in the presence of the test compound is about two-fold over the same region in the control. By contrast, there is a partial inhibition (about 36%) of full length 31-mer product in presence of **4**. Considering the fact that this analogue has also a very weak binding affinity at non-bulge regions (binding constant 20-fold higher than that with the bulge) the inhibititon of full length product is likely to be the sum of a partial block of synthesis at the bulge site and inhibition at other regions of very low affinity binding. There is no evidence that the analogues act on the enzyme itself. On the contrary, they stimulated slippage synthesis at limiting enzyme levels (19). This, combined with the earlier finding that another spirocyclic bulge-binder (DDI) stimulated slippage synthesis, but did not inhibit DNA polymerase-dependent primer extension on single-stranded M13 DNA template (18) further support the conclusion that **4** is not directly acting on the enzyme.

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The enhancement of band intensities at 21–23 in the presence of **4** depended also on the primer length which was varied from 10 to12 nucleotides (Table 1, entries 6–8) . Maximal increase (70–100%) was obtained with the 10-mer primer. As the length of the primer was increased to 11 and 12 nucleotides the enhancement of band intensities at the bulge region decreased significantly. It is likely that duplex formation with the longer primers distorts or opens up the bulge structure so as to interfere with the binding of **4** and/or the stability of its complex. Furthermore, of the three analogues (**2**, **3**, **4)**, **2** was the least efficient in inhibiting primer extension and in enhancing band intensities at the bulge region, a pattern reflecting their relative bulge-binding efficiencies obtained in fluorescence-based assays (data not shown). In addition, dose-response experiments for **4** gave an inhibition of 25% and 56% for full length product, respectively, at 50 μM and 100 μM levels with a concomitant increase in band intensities in the bulge region. These levels of **4** were also quite effective in cleavage inhibition as shown in Figure 4. From these results it is reasonable to conjecture that the increase in band intensities at sites spanning the bulge is the result of a slowing down of the synthesis due to the presence of the bulge-binder and a consequent stabilization of the complex. The finding, that the block at positions 21–23 is only partial, is not unexpected since the binding is noncovalent in contrast to the nearly total inhibition obtained in synthesis on the 31-mer template having a covalently bound NCS-chrom adduct (23).

Additional support for the notion that the enhancement of band intensities at 21–23 region is due to the bulge-bound **4** comes from experiments where the extension of the same 5'-^{32}P-end-labeled 10-mer was compared using as templates, the 31-mer and a 29-mer derived from it by deletion of the two-base bulge (Figure 6A and 6B) . A gel profile of the synthesis products with the two templates is shown in Figure 8. In synthesis with the 31-mer template addition of **4** caused an increase in band intensities at 21–23 of the putative bulge region (lanes 3, 4, arrow) when compared to the same region in the controls lacking it (lanes 1, 2). By contrast, in reactions using the 29-mer as template (lanes 5–8) addition of **4** did not cause an increase in band intensity (lanes 7, 8) at any position including that from where the 2-base bulge was deleted. Instead, in the presence of **4** there is an overall small level of inhibition which, as explained for the 31-mer, may result from weak binding of the compound at non bulge sites. In summary, the results obtained in the polymerase assays show that even the noncovalently bound bulge-specific compounds can modulate DNA synthesis on templates containing putative bulges.

CONCLUSION

Previously reported synthetic analogues (15-17), modeled after the natural metabolite **1** of NCS-chrom, showed selective binding affinity for DNA bulges and also stimulated DNA slippage synthesis, presumably, by binding to or inducing the formation of bulge intermediates involved in the process itself. Nevertheless, their bulge-binding affinity was significantly less than that of the natural metabolite **1**. Further, these analogues, possessing the aminosugar moiety on the spirocyclic ring rather than on the 5-membered ring akin to the natural product, have been shown by 2-D NMR (24,25) to intercalate incompletely at the 2-base bulge site and do so via the minor groove. Of the three compounds in this study **4** is the closest in structure to the natural metabolite **1** in having N-methylfucosamine in an α- glycosidic linkage. Recent 2-D NMR studies have shown that compound **4** closely mimics the natural product in intercalating completely at the 2-base bulge site via the major groove (N. Zhang and I. H. Goldberg, unpublished data). The finding that **4** can effectively block bulge-specific strand cleavage by NCS-chrom and slow down DNA synthesis selectively at the bulge site shows the potential usefulness of bulge-binding compounds in biological studies pertaining to DNA structure and function and suggest that a reactive (e.g., alkylating) species of the analogues will bind covalently at the bulge and interfere directly with its involvement in various biological processes.

Acknowledgements

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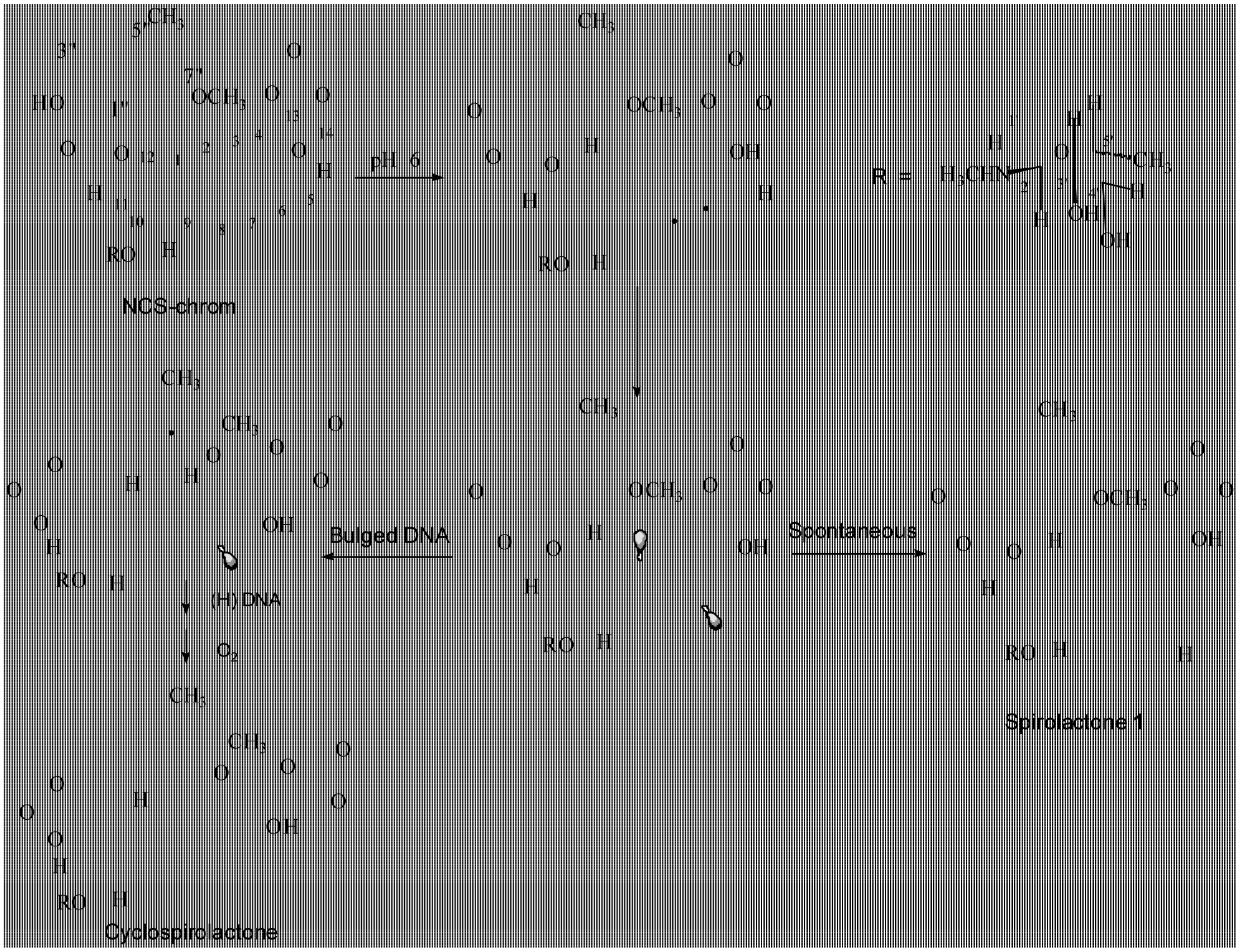
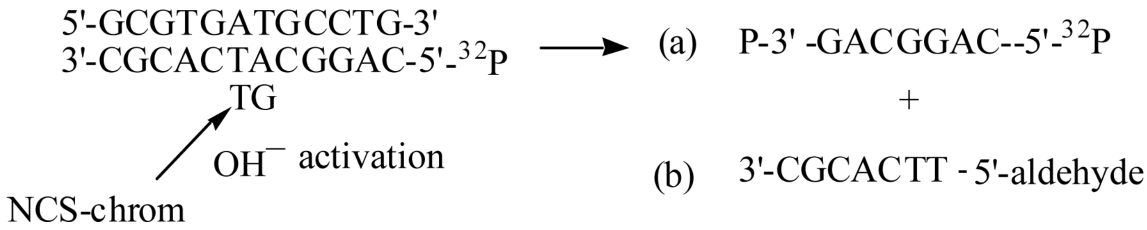
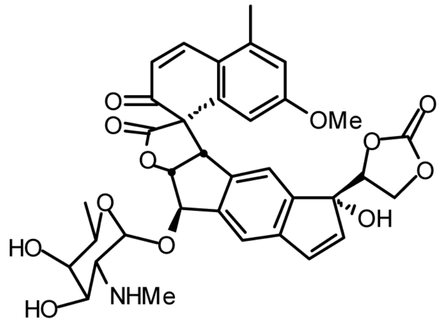


Scheme 1.
Proposed mechanism for base-catalyzed NCS-chrom activation and DNA damage.

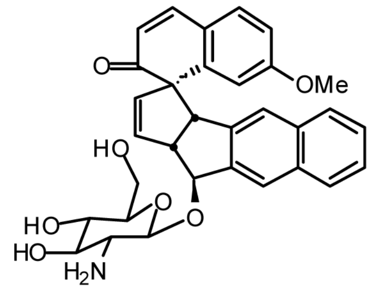


Scheme 2.

Illustration of strand scission by NCS-chrom at a 2-base bulge in a DNA duplex. Arrow points to the target T. Cleavage results in two fragments: (a) having ^{32}P label at its 5’ end and phosphate at the 3’ end and (b) the unlabeled product, has a nucleoside 5’ aldehyde.



(NCS-chrom metabolite) **1**



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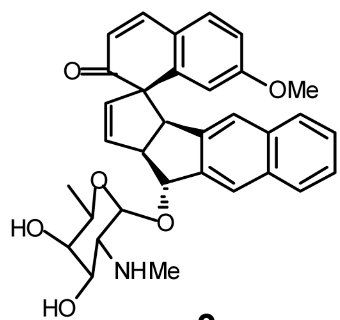

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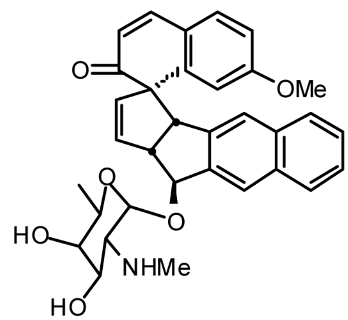

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Figure 1.

Structures NCS-chrom metabolite **1** (spirolactone **1** in Scheme 1) and synthetic analogues of **1**. **1** has N-methylfucosamine in α- glycosidic linkage; **2–4** are synthetic analogues of **1**. Analogue **2** has aminoglucose in β- glycosidic linkage; **3** and **4** have N-methylfucosamine in α− glycosidic linkage.

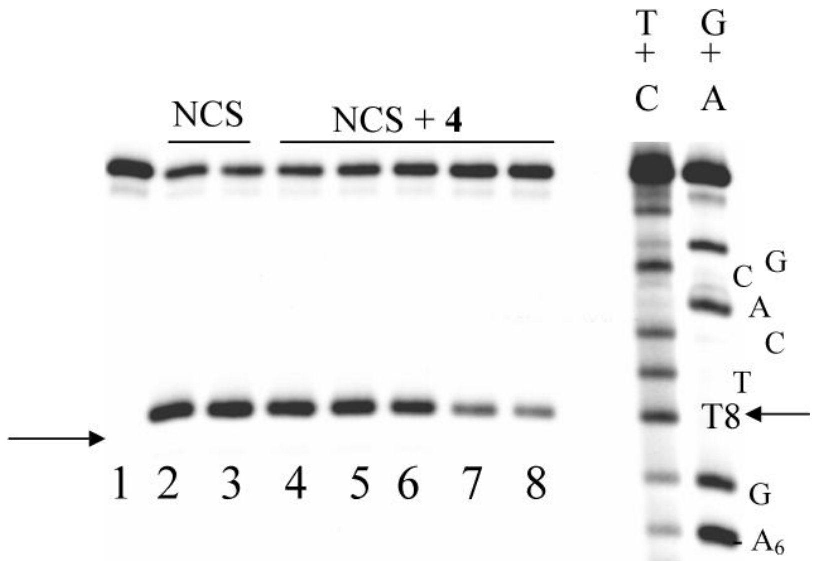

Figure 2.

Effect of compound **4** on strand scission at a 2-base bulge in a DNA duplex by NCS-chrom. In a standard cleavage reaction (40 min) containing the bulge duplex 1 of Table 1 (5.5 μM) having 5’-^{32}P-end label on the bulge strand was treated with NCS-chrom in the absence and presence of varying amounts of **4** as described in Materials and Methods. A gel analysis profile of the products is shown. Lane 1, control duplex DNA without any treatment; lanes 2 and 3, duplicates of DNA treated with only NCS-chrom (15 μM)) ; lanes 4−8, with NCS-chrom in the presence of **4** at concentrations of 10, 20, 40, 80, and 166 μM, respectively. Lanes T+C and G+A show Maxam-Gilbert markers made from the 5’-^{32}P-end-labeled bulge strand. Arrow points to the target site T8.

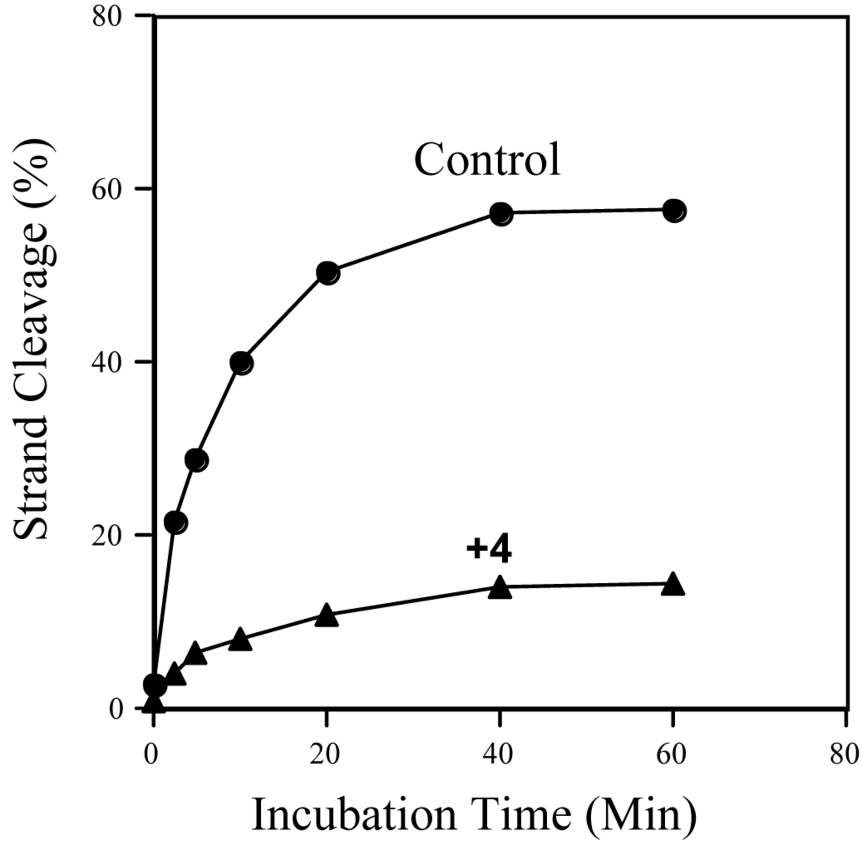

Figure 3.
Time course of inhibition of NCS-chrom-induced strand scission at the bulge by **4**. In experiments similar to those in Figure 2, bulge duplex 2 of Table 1(10 μM) having 5'-^{32}P-end label on the 10-mer strand was treated with NCS-chrom (21 μM). When present, **4** was at a concentration of 150 μM. At times indicated, aliquots were withdrawn to stop the reaction. After gel separation of the products, the gel band intensities were quantitated.

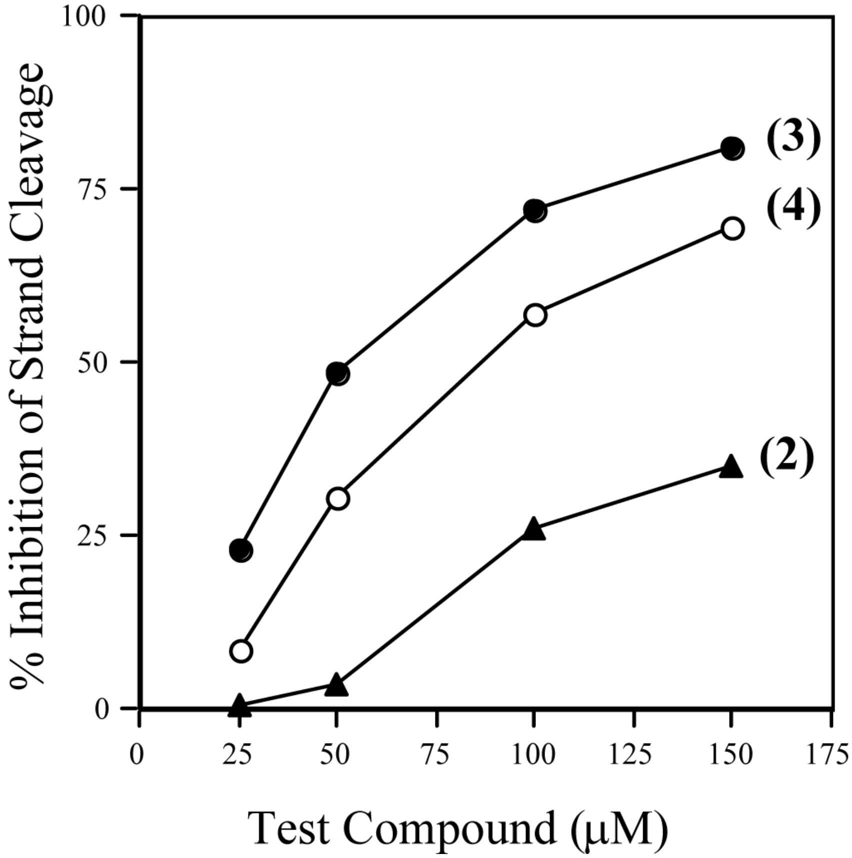

Figure 4.
Dose response for analogues **2**, **3**, and **4** in the inhibition of bulge-specific cleavage by NCS-chrom (20 μM): Strand cleavage reactions similar to those in Figure 3 were performed using 5’-^{32}P-end-labeled bulge duplex in the absence and presence of varying amounts of the indicated analogues. After separation of the products on a sequencing gel, the band intensities were quantitated.

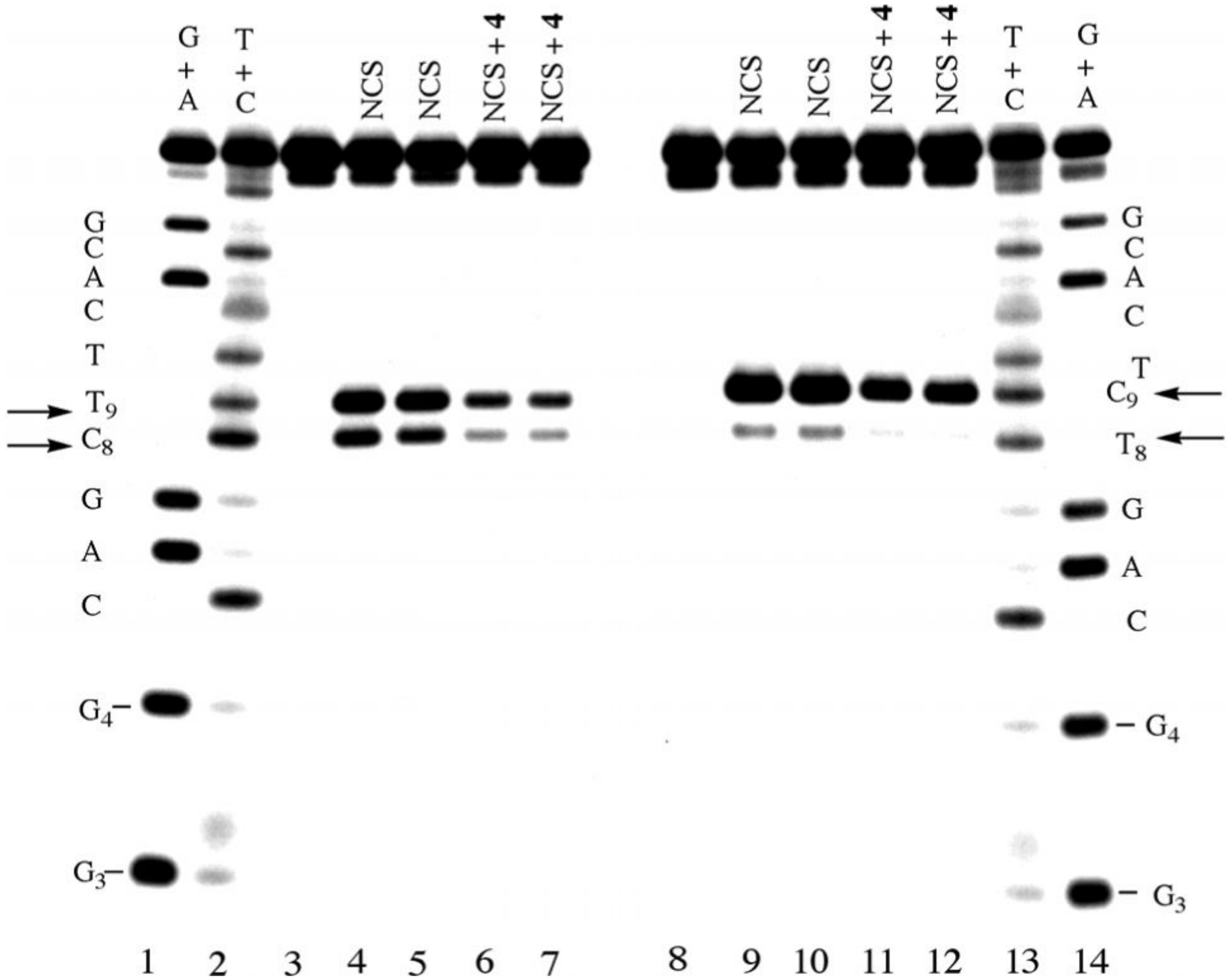

Figure 5.

Effect of **4** on strand scission at 3-base bulges in DNA. Duplexes 3 and 4 (5 μM each) of Table 1 having ^{32}P label on the 5' end of the bulge strand were treated for 30 min with NCS-chrom (45 μM) in the absence and presence of compound **4** (67 μM). A gel profile of the reaction products is shown. Lanes 1−7 represent DNA duplex 3. Lane 3, control DNA without any addition; lanes 4 and 5, duplicates of DNA treated with NCS-chrom; lanes 6 and 7, duplicates of DNA treated with NCS-chrom in the presence of compound **4**. Lanes 8−14 represent DNA duplex 4. Lane 8, control DNA without any addition; lanes 9 and 10, DNA treated with NCS-chrom; lanes 11 and 12, DNA treated with NCS-chrom in the presence of compound **4**. Lanes T+C and G+A show Maxam-Gilbert markers made from the 5'-^{32}P-end labeled bulge strands. Arrows indicate the bulge site of cleavage.

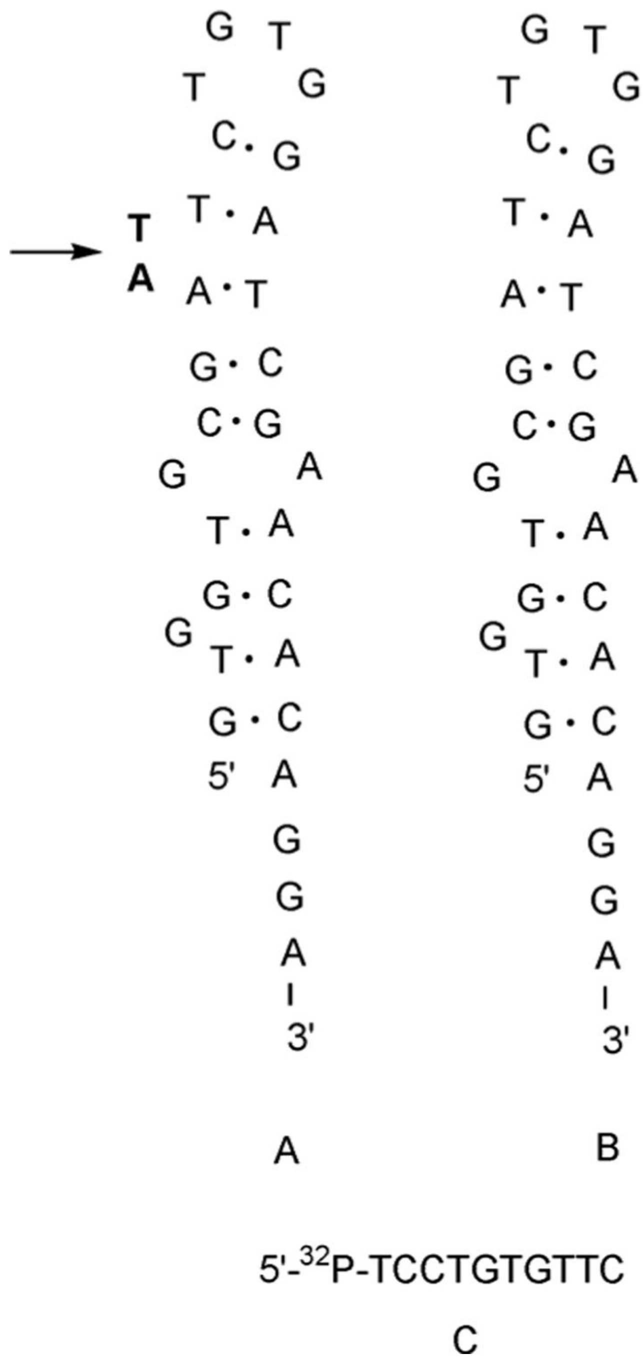

Figure 6.
Putative folding pattern of: A. 31-mer (Table 1, entry 9) and B. 29-mer (Table 1, entry 10). Arrow and the **bold** letters indicate the 2-base bulge. C is the 10-mer primer that was annealed to the 3’-end of the 31-mer and the 29-mer in polymerase reactions.

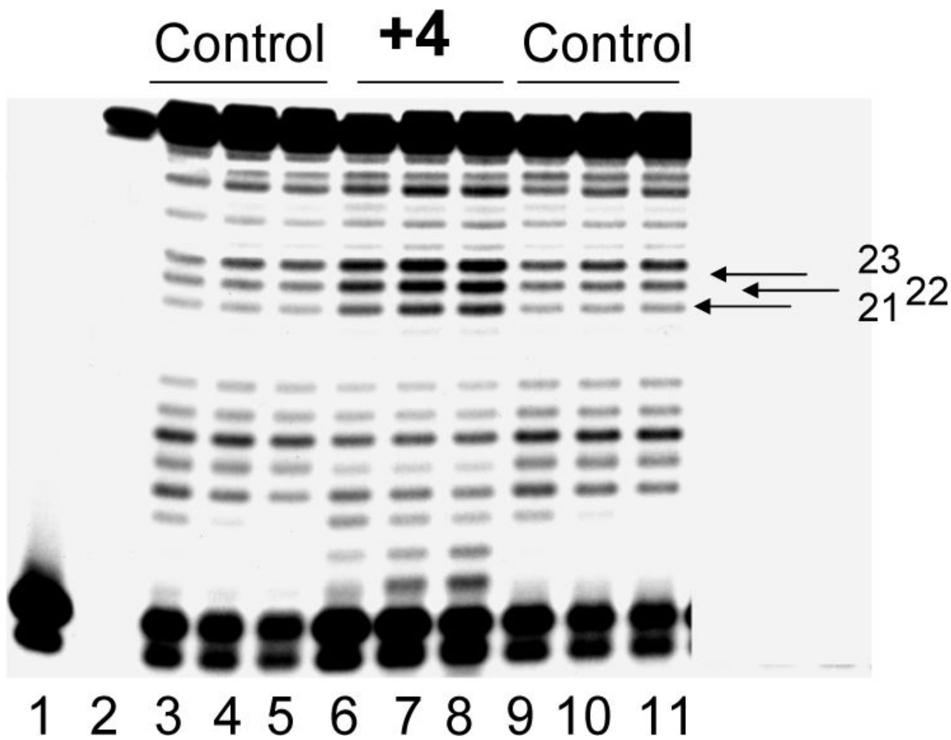

Figure 7.
Effect of **4** on DNA polymerase-dependent primer extension (time course) on a template containing a putative 2-base bulge. 5’-^{32}P-end-labeled 10-mer primer was annealed to the 31-mer (Figure 6A). Its extension by DNA polymerase at various times was followed, in the absence and presence of **4** (100 μM) as described in Materials and Methods. Samples were analyzed on a sequencing gel. Lanes 3, 4, 5 and 9, 10, 11 are duplicate controls lacking the test compound and lanes 6, 7 and 8 represent reactions containing the test compound at 15, 45 and 90 min of incubation, respectively. Arrows indicate the length of the synthesis products at the bulge region of the template.

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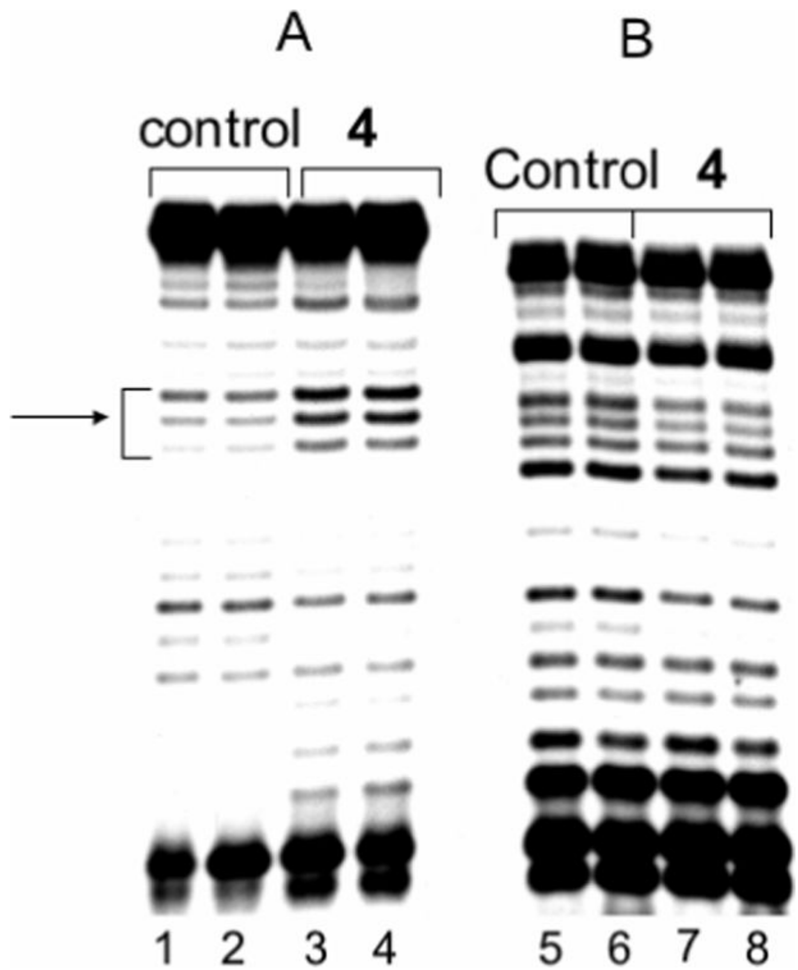

Figure 8.

Effect of **4** on primer extension on a DNA template containing or lacking a bulge. In experiments similar to those in Figure 7, 5'-^{32}P-end-labeled 10-mer was extended on the 31-mer template (lanes 1–4) and on the 29-mer derived from it by deletion of its 2-base bulge (lanes 5–8). Lanes 1 and 2 are control reactions for 1 h and 2 h respectively; lanes 3 and 4, reactions in the presence of test compound **4** for 1 h and 2 h respectively. Similarly, lanes 5 and 6 have control reactions for 1 h and 2 h, and lanes 7 and 8 represent those with the test compound for 1 h and 2 h respectively. Compound 4 was present at a level of 100 μM. Arrows indicate the bulge region containing products of length 21–23 nucleotides.

Table 1

Oligonucleotide Duplexes and DNA Templates and Primers[a]

1.	5′-GCGTGATGCCTG-3′ 3′-CGCACTACGGAC-5′ TG
2.	5′-GCGATGCC-3′ 3′-CGCTACGG-5′ TG
3.	5′-GCGTGATGCCTG-3′ 3′-CGCACTACGGAC-5′ TG C
4.	5′-GCGTGATGCCTG-3′ 3′-CGCACTACGGAC-5′ CG T
5.	5′-GCGAACTGCC-3′ 3′-CGCTTGACGG-5′
6.	5′-TCCTGTGTTC
7.	5′-TCCTGTGTTCG
8.	5′-TCCTGTGTTCGA
9.	5′-GTGGTGCGAATTCTGTGGATCGAACACAGGA-3′
10.	5′-GTGGTGCGATCTGTGGATCGAACACAGGA-3′

[a]The duplexes represent the annealed mixtures of the indicated oligomers.

Table 2

Effect Of **4** on DNA synthesis involving DNA polymerase-dependent primer extension on bulge templates[a].

Incubation (Min)	% Increase of Band Intensity at the Bulge Site	% Inhibition of Full Length Product
15	60	36
45	100	36
90	105	30

[a]The intensities of bands at 21−23 and that of the full length product in experiments shown in Figure 7 were quantitated.