Alteration of DNA Primary Structure by DNA Topoisomerase I. Isolation of the Covalent Topoisomerase I−DNA Binary Complex in Enzymatically Competent Form

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Abstract: DNA ligation by DNA topoisomerase I was investigated employing synthetic DNA substrates containing a single strand nick. Site-specific cleavage of the DNA by topoisomerase I in proximity to the nick resulted in uncoupling of the cleavage and ligation reactions of the enzyme, thereby trapping the covalent enzyme−DNA intermediate. DNA cleavage could be reversed by the addition of acceptor oligonucleotides containing a free 5′-OH group and capable of hybridizing to the noncleaved strand of the "suicide substrates". Utilizing acceptors with partial complementarity, modification of nucleic acid structure has been obtained. Modifications included the formation of DNA insertions, deletions, and mismatches. To further evaluate the potential of topoisomerase I to mediate structural transformations of DNA, acceptor oligonucleotides containing nucleophiles other than OH groups at the 5′-end were studied as substrates for the topoisomerase I-mediated ligation reaction. Toward this end, oligonucleotides containing 5′-thio, amino, and hydroxymethylene moieties were synthesized. Initial investigations utilizing a coupled cleavage−ligation assay suggested that only the modified acceptor containing an additional methylene group underwent efficient enzyme-mediated ligation. However, as linear DNA is not a preferred substrate for topoisomerase I, the enzyme−DNA intermediate was purified to homogeneity, thereby allowing investigation of the ligation reaction independent of the forward reaction that formed the covalent binary complex. The isolated complex consisted of equimolar enzyme and DNA, with topoisomerase I covalently bound to a specific site on the DNA duplex in an enzymatically competent form. Displacement of the enzyme-linked tyrosine moiety of the enzyme−DNA binary complex was effected by all the modified acceptor oligonucleotides, affording unnatural internucleosidic linkages at a specific site. Characterization of the formed linkages was effected both by enzymatic and chemical degradation studies. Comparative analysis revealed overall differences in the efficiency and rate of the topoisomerase I-mediated ligation of the modified acceptors. Moreover, the facility of ligation of the amino acceptor was significantly enhanced at increasing pH values. In addition, the method utilized to obtain the topoisomerase I−DNA intermediate is capable of affording large quantities required for further mechanistic and physicochemical characterization of the formed binary complex.

The DNA topoisomerases control DNA topology through the introduction of transient breaks in the phosphodiester backbone.[1] Owing to their unique function, these enzymes are central to the essential cellular processes of replication and transcription,[2] as well as recombination.[3] DNA topoisomerases are classified into two groups based on the mode of DNA strand scission: the type I enzymes mediate the transient single-strand breakage of duplex DNA, while the type II topoisomerases break both strands of the duplex.

Mechanistically, the transient strand breaks mediated by eukaryotic DNA topoisomerase I involve reversible formation of an intermediate in which the active site of topoisomerase I is linked to the DNA substrate covalently via a 3′-*O*-phosphorotyrosine bond, with concomitant production of a free 5′-OH group on the DNA at the site of the break.[4] DNA ligation is coordinated with cleavage and restores continuity to the DNA duplex. Under normal circumstances the cleavage and ligation reactions of the enzyme are tightly coupled, with a low steady-state concentration of the covalent intermediate.[5] However, the two half-reactions of topoisomerase I can be separated *in vitro* by site specific cleavage of partial duplex substrates containing a high affinity cleavage site toward the 3′-end of the scissile strand (Figure 1A).[6] Cleavage of the suicide substrate occurs without sequential religation due the instability of the truncated strand downstream from the site of cleavage; loss of this short oligonucleotide traps the topoisomerase I−DNA covalent intermediate. The covalently bound enzyme is catalytically competent; admixture of an acceptor oligonucleotide complementary to the single-stranded region of the covalent binary complex results in ligation to reform a duplex.[6] The ability of topoisomerase I to facilitate the formation of phosphodiester bonds other than those broken in the original transesterification reaction suggests that the enzyme can catalyze covalent alterations in DNA connectivity.[7] In fact, through the use of acceptor strands of varying length and sequence, topoisomerase

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(2) (a) Wang, J. C. *Harvey Lectures* **1987**, *81*, 93. (b) Osheroff, N. *Pharmacol. Ther.* **1989**, *41*, 223.

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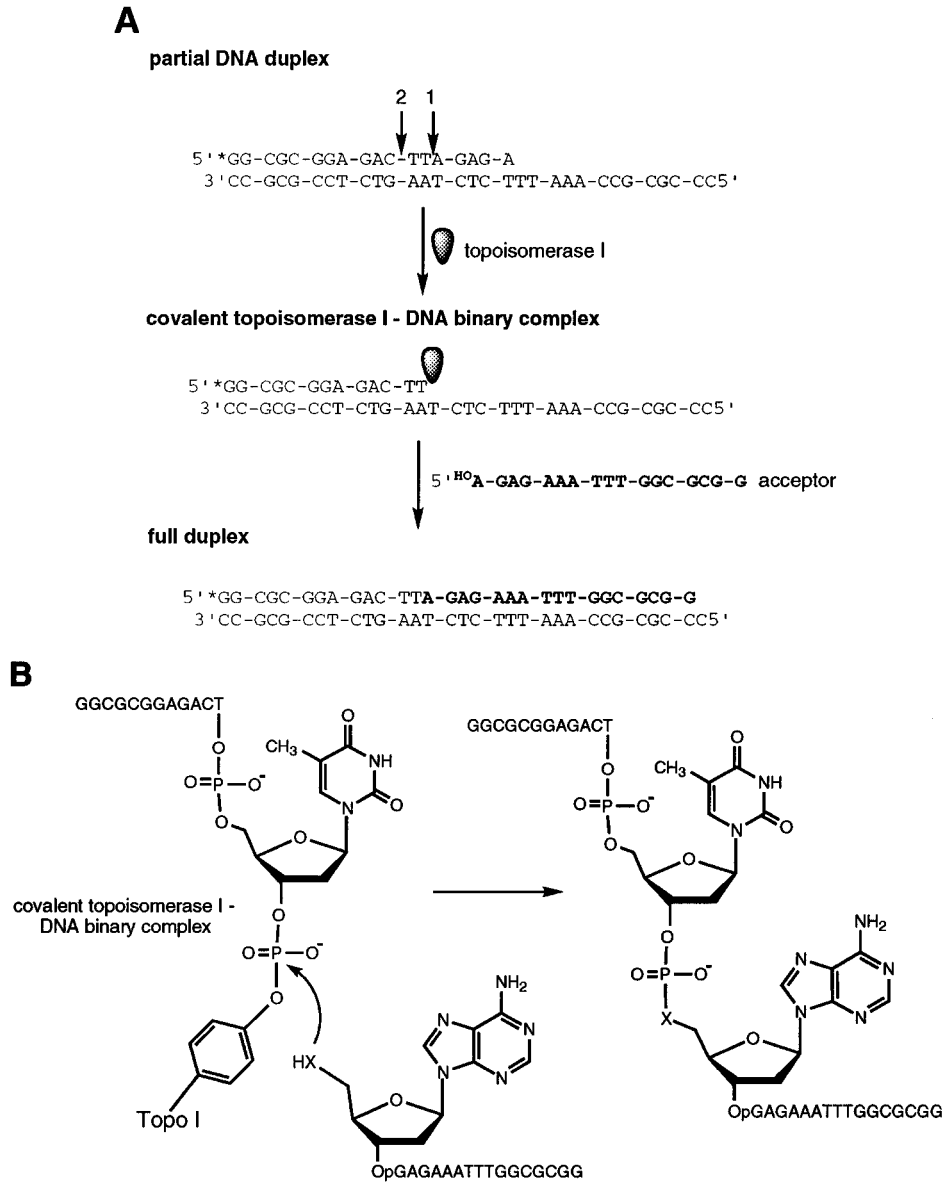

Figure 1. (A) DNA substrate utilized to uncouple the cleavage and ligation reactions of topoisomerase I, thereby allowing DNA strand exchange. **(B)** Topoisomerase I-mediated ligation reaction.

I-mediated DNA insertions, deletions and mismatches have been shown to obtain *in vitro* with quite reasonable efficiency.[8,9]

To further define the capacity of topoisomerase I to catalyze structural transformations of DNA, modified DNA oligonucleotides containing nucleophiles other than the 5′-OH group of deoxyribose were studied as substrates for the ligation reaction. Oligonucleotides containing 5′-OH, -SH, -NH_2 or -CH_2OH functionalities at their 5′-termini were prepared and the ability of these acceptors to undergo topoisomerase I-mediated ligation was investigated both in a coupled cleavage−ligation assay, and also employing the purified topoisomerase I−DNA covalent binary complex (Figure 1B). Presently, we describe the purification of the topoisomerase I−DNA covalent binary complex and the chemical synthesis of the nucleoside analogues which permitted the study of the reverse reaction in the presence of several different nucleophilic functionalities. All modified oligonucleotide acceptors studied were capable of displacing the covalently bound topoisomerase I with concomitant alteration in the internucleosidic bond and release of free topoisomerase I. These results further expand the repertoire of topoisomerase I-catalyzed nucleic acid rearrangements.

Results

Synthesis of 5′-Homo-2′-deoxyadenosine. Although several methods have been described for the preparation of homoadenosine[10] and the corresponding thymidine analogue,[11] the application of these procedures for preparative purposes in this study proved to be problematic owing to poor yields and side reactions. The route employed for the synthesis of the 5′-homologue of deoxyadenosine is outlined in Scheme 1. Benzylation of α-D-allofuranose **5** gave benzyl ether **6** in quantitative

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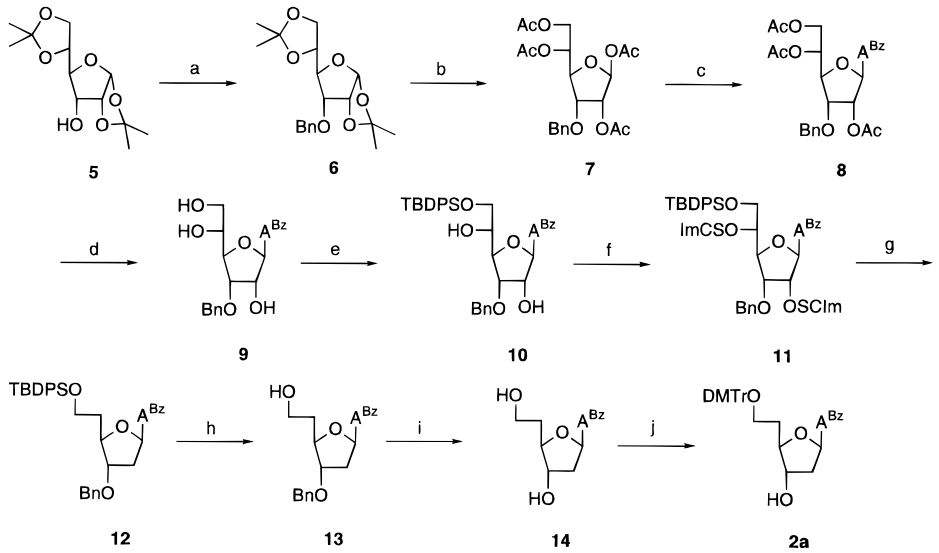
(10) (a) Ryan, K. J.; Arzoumanian, H.; Acton, E. M.; Goodman, L. *J. Am. Chem. Soc.* **1964**, *86*, 2503. (b) Pfitzner, K. E.; Moffatt, J. G. *J. Am. Chem. Soc.* **1965**, *87*, 5661. (c) Kappler, F.; Hampton, A. In *Nucleic Acid Chemistry, Part 4*; Townsend, L. B., Tipson, R. S. Eds.; Wiley Interscience, New York, 1991, p 240.
(11) (a) Lebreton, J.; De Mesmaeker, A.; Waldner, A.; Valerie, F.; Wolf, R. M.; Freier, S. M. *Tetrahedron Lett.* **1993**, *34*, 6383. (b) Wendeborn, S.; Wolf, R. M.; De Mesmaeker, A. *Tetrahedron Lett.* **1995**, *36*, 6879.

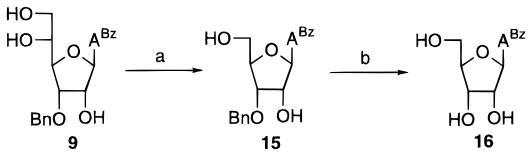
Scheme 1[a]



[a] (a) BnBr, NaH, DMF, quantitative; (b) Ac_2O, AcOH, TsOH, 97%; (c) BisTMS-A^{Bz}, TMSOTf, CH_3CN, 71%; (d) 2 N NaOH, EtOH−pyridine, 86%; (e) TBDPSCl, imidazole, CH_2Cl_2, 97%; (f) Im_2CS, THF, 65 °C, 90%; (g) Bu_3SnH, AIBN, toluene, 75 °C, 80%; (h) TBAF, THF, 76%; (i) Pd black, EtOH, 45 °C, 9 h, 83%; (j) DMTrCl, pyridine, 81%.

yield. The diacetonide (**6**) was then hydrolyzed and protected as the tetra-*O*-acetate by treatment with acetic anhydride in the presence of a catalytic amount of *p*-toluenesulfonic acid in AcOH, which afforded **7** in 97% yield as a colorless solid. A diastereomeric ratio of 9:1 was obtained, with the β-anomer presumably being the major isomer.[12,13] Initial attempts to introduce a bis-TMS-N^6-benzoyladenine moiety into tetraacetate **7** under standard Vorbrüggen conditions[14] at room temperature using 1 equivalent of trimethylsilyl trifluoromethanesulfonate (TMSOTf) as a Lewis acid were unsuccessful. However, a 71% yield of nucleoside **8** was obtained if the condensation was performed at 55 °C in the presence of 0.28 equivalent of TMSOTf. Selective removal of the acetyl groups of **8** was effected by treatment with 2 N NaOH in ethanol-pyridine to afford triol **9** in 86% yield.[15] Protection of the primary hydroxyl group of **9** as a silyl ether gave **10** in 97% yield. Subsequent thioacylation of the free alcohols with thiocarbonyldiimidazole in THF afforded **11** as a colorless powder in 90% yield.[16] Deoxygenation of nucleoside **11** via the Barton procedure[17] using tributyltin hydride and azo-bis-isobutyronitrile (AIBN) furnished **12** in 80% yield. The silyl group was removed utilizing TBAF in THF to give **13** in 76% yield. Debenzylation with Pd black in ethanol[18] afforded the corresponding 5′-homo-2′-deoxyadenosine (**14**) as a colorless powder in 83% yield. The primary hydroxyl group of **14** was protected with dimethoxytrityl chloride in pyridine to furnish the 5′-DMTr-homodeoxyadenosine **2a** in 81% yield.

Scheme 2[a]



[a] (a) $NaIO_4$, THF-H_2O, $NaBH_4$, MeOH; (b) Pd black, EtOH, 45 °C, 9 h.

Verification of the anomeric configuration of this nucleoside analogue was obtained by the conversion of triol **9** to N^6-benzoyladenosine as outlined in Scheme 2. Oxidative cleavage of the diol moiety of **9** with sodium periodate[19] and subsequent reduction of the derived aldehyde with $NaBH_4$ (to prevent epimerization at C4′)[20] afforded adenosine derivative **15** in 83% overall yield. Debenzylation of **15** was effected utilizing palladium black to furnish N^6-benzoyladenosine (**16**) in 68% yield. The ^{1}H NMR spectrum and the optical rotation of synthetic nucleoside **16** were identical to those of an authentic sample of **16**.

Synthesis of 5′-Thio-2′,5′-dideoxyriboadenosine. The synthesis of the 5′-thio-2′-deoxyadenosine derivative (**3a**) is outlined in Scheme 3. The 5′-hydroxyl group of 2′-deoxyadenosine (**17**) was converted to the thioacetate via the Mitsunobu reaction[21] using PPh_3, DEAD and thioacetic acid in THF to furnish **18** in 88% yield.[22] Treatment of **18** with benzoyl chloride gave the N^6-bis benzoyl protected nucleoside **19** in 92% yield. The ^{1}H NMR ratio of *ortho* proton signals of the benzoyl groups of the amide relative to the *ortho* proton signals of the benzoyl ester, as well as the absence of an amide proton signal, provided structural evidence for nucleoside **19**. Thio ester **19** was treated with 2 N NaOH to afford the desired modified nucleoside **20** in 90% yield. The amide proton signal at 8.92 ppm in the ^{1}H

(12) Huang, Z.; Schneider, K. C.; Benner, S. A. *J. Org. Chem.* **1991**, *56*, 3869.

(13) Under the same reaction conditions the 3-*O*-TBDPS-protected diacetonide gave the corresponding tetraacetate as a 1:1 diastereomeric mixture.

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(16) (a) Rasmussen, J. R.; Slinger, C. J.; Kordish, R. J.; Newman-Evans, D. D. *J. Org. Chem.* **1981**, *46*, 4843. (b) Robins, M. J.; Wilson, J. S. *J. Am. Chem. Soc.* **1981**, *103*, 932. (c) Lessor, R. A.; Leonard, N. J. *J. Org. Chem.* **1981**, *46*, 4300.

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(18) Felix, A. M.; Jimehez, H. M.; Meienhofer, J. *Org. Syn.* **1979**, *59*, 159.

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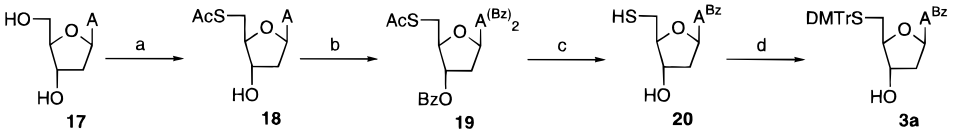
(20) Ranganathan, R. S.; Jones, G. H.; Moffatt, J. G. *J. Org. Chem.* **1974**, *39*, 290.

(21) (a) Mitsunobu, O. *Synthesis* **1981**, 1.

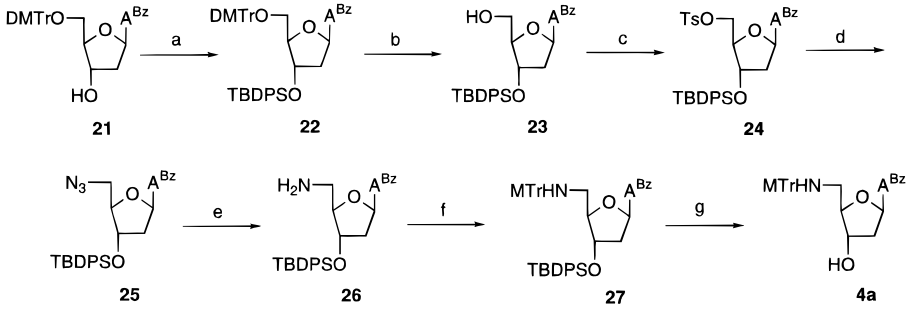
(22) (a) Hugles, D. L. *Org. React.* **1992**, *42*, 335. (b) Dodge, J. A.; Trujillo, J. I.; Presnell, M. *J. Org. Chem.* **1994**, *59*, 234.

Scheme 3[a]



[a] (a) AcSH, PPh_3, DEAD, THF, 88%; (b) BzCl, pyridine, 92%; (c) 2 N NaOH, EtOH−pyridine, 90%; (d) DMTrCl, pyridine, 91%.

Scheme 4[a]



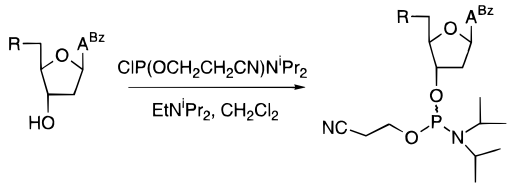
[a] (a) TBDPSCl, imidazole, CH_2Cl_2, 78%; (b) AcOH, 77%; (c) TsCl, pyridine, 85%; (d) NaN_3, DMF, 95%; (e) H_2, Pd on C, EtOH, 80%; (f) MTrCl, pyridine, 91%; (g) TBAF, THF, 81%.

NMR spectrum, as well as the the triplet signal of the SH group at 1.60 ppm and the ratio of protons, indicated that the disulfide had not formed during the reaction and that one of the amide benzoyl groups was hydrolyzed under the reaction conditions. Loss of the benzoyl group was also confirmed by mass spectrometry. Selective protection of the 5′-thiol moiety of **20** with DMTrCl afforded the thio ether **3a** as a colorless foam in 91% yield.

Synthesis of 5′-Amino-2′,5′-dideoxyadenosine. The synthesis of the 5′-amino nucleoside **4a** was carried out in analogy with a previously reported synthesis.[23] The 5′ protecting group was changed to a lipophilic, acid-labile group, thereby allowing reverse phase purification of the modified oligonucleotide prepared using monomer **4b**, following removal of the base and phosphorous protecting groups. The preparation of 5′-amino-2′, 5′-dideoxyadenosine is illustrated in Scheme 4. 5′-O-DMTr-N^6-benzoyl-2′-deoxyadenosine (**21**) was protected as the silyl ether by treatment with TBDPSCl, affording **22** in 78% yield. Following detritylation to afford **23** in 77% yield, the 5′-OH group was activated with tosyl chloride[24] in pyridine to furnish nucleoside **24** in 85% yield. Displacement of the tosylate was effected via the agency of NaN_3 in DMF to give the 5′-azido nucleoside **25** in 95% yield. The 5′-azido group was reduced over 10% palladium-on-carbon by hydrogenation in ethanol, affording the 5′-amino compound **26** in 80% yield. The 5′-amino group of the nucleoside was protected with monomethoxytrityl chloride in pyridine to furnish **27** in 91% yield**.** Treatment with TBAF effected removal of the *tert*-butyldiphenylsilyl group to give **4a** as a colorless foam in 81% yield.

Oligonucleotide Synthesis. Phosphitylation of the nucleoside monomers using 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite and diispropylethylamine in $CHCl_2$ afforded the 3′-*O*-phosphoramidite derivatives **2b-4b** (Scheme 5). The monomers were incorporated at the 5′-termini of the acceptor oligonucleotides using standard solid phase phosphoramidite chemistry.[25] Cleavage from the solid support and removal of the base and phosphate protecting groups was effected with concentrated NH_4OH for 12 h at 55 °C. The DMTr and MTr-containing

Scheme 5



2a R = DMTrCH$_2$O
3a R = DMTrS
4a R = MTrHN

1b R = DMTrO
2b R = DMTrCH$_2$O
3b R = DMTrS
4b R = MTrHN

oligonucleotides were purified and the trityl group was then cleaved with trifluoroacetic acid using Nensorb Prep chromatography to isolate **II** and **IV**. During oligonucleotide synthesis the thiol-linked DMTr group was removed, presumably as a consequence of iodine treatment, resulting in the formation of the disulfide-containing oligonucleotide. This dimeric oligonucleotide was purified on a polyacrylamide gel. Subsequent reduction with dithiothreitol (DTT) afforded the desired 5′-thiol-containing product (**III**). Loss of the DMTr moiety most likely occurred during the oxidation step of DNA synthesis as *S*-trityl cleavage by iodine has previously been shown to occur via intermolecular disulfide bond formation.[26] Interestingly, another report describes the preparation of an oligonucleotide containing a modified *S*-trityl moiety at the 5′-terminus that was stable toward iodine oxidation during oligonucleotide synthesis.[27]

Topoisomerase I Coupled Cleavage−Ligation. The 5′-^{32}P end labeled partial duplex shown in Figure 1A was treated with calf thymus DNA topoisomerase I in the presence of an excess of the acceptor oligonucleotide at 37 °C for 60 min. Following proteolysis to remove the covalently bound enzyme, the reactions were analyzed on a 20% denaturing polyacrylamide gel. The acceptor oligonucleotide containing a 5′-OH group (**I**) afforded a single ligated product (supporting information, Figure 1). DNA sequence analysis of the isolated product verified that the ligation product resulted from cleavage of the partial duplex at site 1, with concomitant loss of the pentanucleotide 5′-AGAGA-3′, and ligation of the acceptor strand to the truncated DNA (supporting information, Figure 2). As

(23) Sproat, S. B.; Beijer, B.; Rider, P. *Nucleic Acids Res.* **1987**, *15*, 6181.
(24) Reist, E. J.; Benitez, A.; Goodman, L. *J. Org. Chem.* **1964**, *29*, 554.
(25) Caruthers, M. H. *Science* **1985**, *230*, 281.
(26) (a) Kamber, B.; Rittel, W. *Helv. Chim. Acta* **1968**, *51*, 2061. (b) Kamber, B. *Helv. Chim. Acta* **1971**, *54*, 398.
(27) Connolly, B. A. *Nucleic Acids Res.* **1985**, *13*, 4485.

described previously, the presence of the acceptor strand complementary to the single-stranded region downstream from the intended site of cleavage promoted cleavage at an additional site two nucleotides upstream from the intended site of cleavage, i.e. site 2 (cf. Figure 1A).[8] When topoisomerase I-mediated cleavage and ligation were performed in the presence of the modified acceptor oligonucleotide **II**, significantly lower levels of ligation were observed compared with acceptor **I** (supporting information, Figure 1, cf lanes 2 and 4). However, the topoisomerase I−covalent intermediate was formed at both sites, as demonstrated by the cleavage products observed.

While the modified acceptor oligonucleotide underwent ligation to a significant extent, complete release of the topoisomerase covalently bound at site 1 was not observed. Attempts to increase the ligation yields of duplex in the presence of acceptor **II** employed agents that facilitate steric exclusion or DNA aggregation, such as polyethylene glycol, spermidine or hexammine cobalt chloride; however, these increased the ligation yields only marginally (data not shown). Moreover, a temperature profile of the course of the ligation reaction simply paralleled the thermostability of the enzyme suggesting that the native conformation of the enzyme is uniquely required for catalysis (data not shown). As the ligation product incorporating acceptor oligonucleotide **I** was formed only to the extent of 34% in the coupled cleavage−ligation assay, less efficient transformations leading to structurally altered duplexes would not have been detected readily utilizing this assay system. Moreover, the presence of an acceptor strand has been shown to influence both the efficiency and the specificity of initial topoisomerase I-mediated cleavage.[8] Thus, to investigate the ligation reaction independent of the forward reaction, the topoisomerase I−DNA covalent intermediate was purified.

Purification of the Topoisomerase I−DNA Covalent Intermediate. The topoisomerase I−DNA covalent intermediate was formed by site-specific cleavage of the nicked duplex shown in Figure 2A. The addition of a 12-nt oligomer (5′-pAATTTGGCGCGG-3′) complementary to the single-stranded region of the partial duplex was introduced to increase the efficiency and specificity of cleavage by topoisomerase I.[8] The 5′-termini of all the oligomers used to assemble the substrate were phosphorylated to prevent ligation with the formed enzyme−DNA binary complex.

The substrate partial duplex, 5′-^{32}P end labeled on the scissile strand, was treated with topoisomerase I for 60 min at 37 °C. Separation of the covalent topoisomerase I−DNA intermediate from both the unreacted DNA substrate and topoisomerase I was achieved utilizing anion exchange chromatography on a Mono Q column. The reaction mixture was applied to a Mono Q column then washed with a linear gradient of aqueous NaCl. As shown in Figure 2B, the DNA substrate is strongly retained on this column; preincubation with topoisomerase I resulted in the formation of a new, early eluting peak. Control experiments demonstrated that topoisomerase I alone did not bind to this matrix.

To determine if the peak formed in the presence of topoisomerase I actually contained topoisomerase I covalently linked to the DNA, the isolated complex was analyzed by 20% denaturing polyacrylamide gel electrophoresis (PAGE). It has been reported previously that topoisomerase I retains DNA to which it is linked covalently in the wells of denaturing polyacrylamide gels.[28] The putative enzyme−DNA complex enters the gel only upon incubation with proteinase K, demonstrating that all of the radiolabeled DNA is covalently bound

(28) Christiansen, K.; Bonven, B. J.; Westergaard, O. *J. Mol. Biol*. **1987**, *193*, 517.

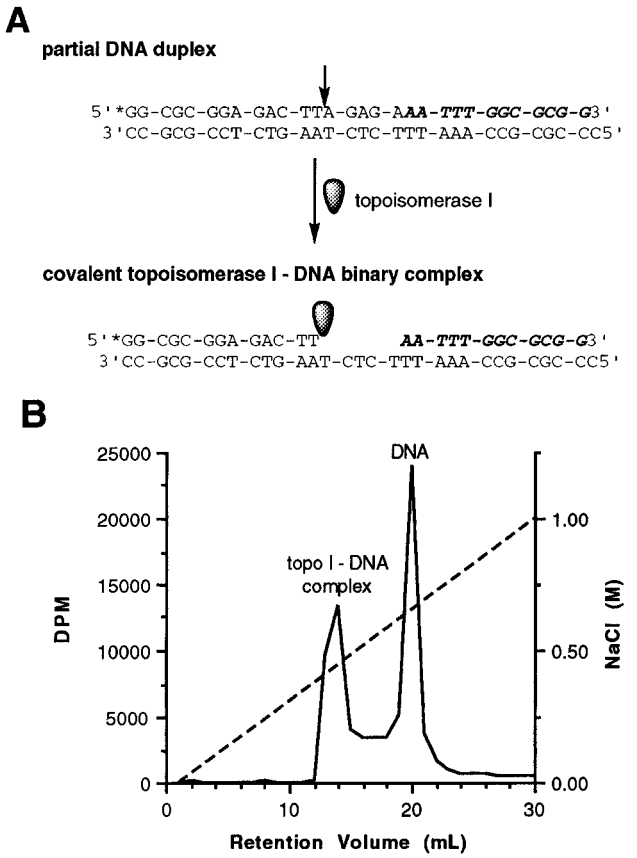


Figure 2. (A) DNA substrate utilized to obtain the covalent topoisomerase I−DNA intermediate. The oligonucleotide which completes the "duplex" is indicated in ***bold italics.*** **(B)** Elution profile showing products formed by treatment of the 5′-^{32}P end labeled partial duplex with topoisomerase I, followed by chromatography on a Mono Q column.

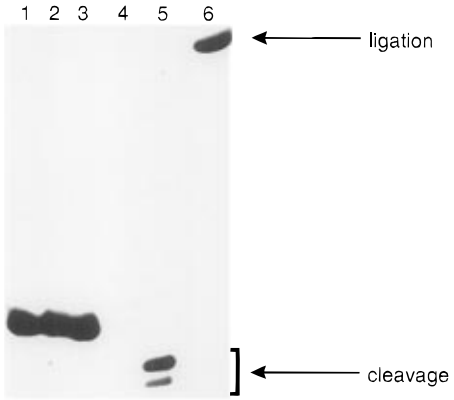


Figure 3. Autoradiogram of a 20% denaturing polyacrylamide gel illustrating the enzymatic activity of the purified topoisomerase I−DNA intermediate: lane 1, DNA alone; lane 2, DNA + proteinase K; lane 3, DNA + **I**; lane 4, putative topoisomerase I−DNA complex alone; lane 5, putative topoisomerase I−DNA complex + proteinase K; lane 6, putative topoisomerase I−DNA complex + **I**.

to protein (Figure 3, cf lanes 4 and 5). Treatment with the protease afforded a DNA fragment with increased electrophoretic mobility compared with the original uncleaved DNA substrate (Figure 3, cf lanes 1 and 5). These results are consistent with topoisomerase I cleavage of the DNA substrate, concomitant formation of the covalent intermediate and subsequent loss of the pentanucleotide downstream from the cleavage site. As expected, the electrophoretic mobility of the DNA substrate itself was not altered by treatment with proteinase K (cf. lanes 1 and 2).

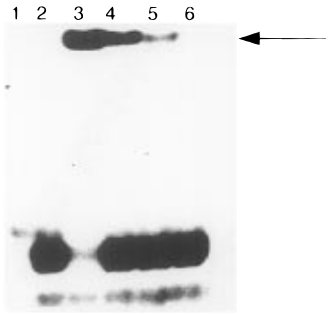


Figure 4. Autoradiogram of a 20% denaturing polyacrylamide gel illustrating the ligation of the modified acceptor oligonucleotides to the 5′-^{32}P end labeled topoisomerase I−DNA intermediate: lane 1, topoisomerase I−DNA complex alone; lane 2, topoisomerase I−DNA complex + proteinase K; lane 3, topoisomerase I−DNA complex + **I**; lane 4, topoisomerase I−DNA complex + **II**; lane 5, topoisomerase I−DNA complex + **III**; lane 6, topoisomerase I−DNA complex + **IV**. Following incubation at 37 °C for 60 min, the reactions in lanes 2−6 were treated with proteinase K prior to analysis. The arrow denotes the position of the ligation products. Although not visible in the gel photograph, the formation of product in the presence of acceptor oligonucleotide **IV** was readily apparent by phosphorimager analysis (cf. Table 1 and supporting information, Figure 3).

Having demonstrated that topoisomerase I was covalently bound to the truncated DNA, the enzymatic competence of the complex was next examined by assaying for strand transfer activity. Incubation of the purified complex with an excess of acceptor oligonucleotide **I** for 60 min at 37 °C effected efficient conversion to a single ligated product (94% yield), demonstrating the enzymatic competence of the covalently bound topoisomerase I (Figure 3, lane 6).

Verification that the duplex structure was maintained during the isolation was accomplished by 5′-^{32}P end labeling the individual strands of the substrate. The purified topoisomerase I−DNA complex was treated with proteinase K and applied to a 20% denaturing polyacrylamide gel confirming the presence of the truncated scissile strand, the 12-mer (5′-AATTTG-GCGCGG-3′) and the noncleaved strand (data not shown).

Topoisomerase I-Mediated Ligation of Modified Acceptors. The topoisomerase I−DNA covalent intermediate, 5′-^{32}P end labeled on the scissile strand, was purified by Mono Q chromatography and then a portion was treated with an acceptor oligonucleotide containing a 5′-OH (**I**), -CH_2OH (**II**), -SH (**III**), or -NH_2 (**IV**) functional group. After 60 min at 37 °C, each reaction was quenched by the addition of 1% sodium dodecyl sulfate (SDS), and the covalently bound enzyme was removed by proteolysis with proteinase K prior to analysis by 20% denaturing polyacrylamide gel electrophoresis. As shown in Figure 4, each of the modified acceptor oligonucleotides (**II**−**IV**) afforded a ligation product that had the same electrophoretic mobility as that obtained with the unmodified acceptor oligonucleotide (**I**). However, the ability of the individual acceptor oligonucleotides to serve as substrates for topoisomerase I-mediated ligation varied. The percent ligation obtained with each acceptor oligonucleotide, relative to unmodified oligonucleotide **I**, was determined by phosphorimager analysis. The results are shown in Table 1. The actual extent of ligation obtained using the unmodified oligonucleotide (**I**) varied from 64−94% in individual experiments. The most effective of the modified oligonucleotides was **II**, containing an additional methylene group at the 5′-end. This species afforded almost 30% ligation product, relative to the unmodified acceptor oligonucleotide. The acceptor oligonucleotide containing an SH group at the 5′-terminus (**III**) gave about 13% ligation product relative to the unmodified acceptor; no difference was observed

Table 1.[a] Quantification of Full Length Ligation Products Resulting from Treatment of the Purified Topoisomerase I−DNA Complex with Acceptor Oligonucleotides **I**−**IV**

acceptor	% ligation	acceptor	% ligation
I	100	**III**	13
II	29	**IV**	4.3

[a] The calculated values were normalized to the ligation obtained with acceptor oligonucleotide **I**.

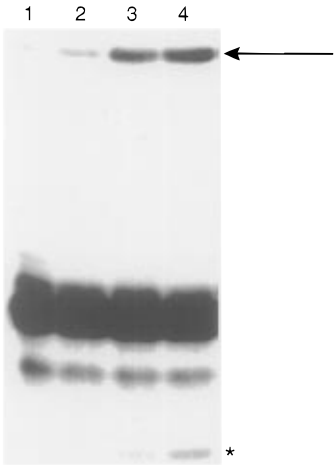
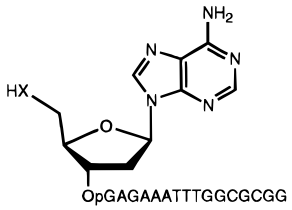


Figure 5. Autoradiogram of a 20% denaturing polyacrylamide gel illustrating the ligation of **IV** as a function of pH: lane 1, pH 7.5; lane 2, pH 8.0; lane 3, pH 8.5; lane 4, pH 9.0. The putative hydrolysis product is indicated by the asterisk.

when Mg^{2+} or Mn^{2+} was employed as the divalent cation (supporting information, Figure 4). The relative efficiency of ligation of the oligonucleotide having a 5′-NH_2 group was only about 4%.

The ability of the individual acceptor oligonucleotides to displace the covalently bound topoisomerase I was also investigated over a range of pH values. Shown in Figure 5 is the topoisomerase I-mediated ligation of acceptor oligonucleotide **IV**



I X = O
II X = OCH_2
III X = S
IV X = NH

(containing a 5′-NH_2 group) in the presence of Tris-HCl at several pH values (7.5, 8.0, 8.5 and 9.0) for 1 h at 37 °C. Phosphorimager quantification (supporting information, Figure 5) clearly demonstrates that as pH value is raised the efficiency of ligation is increased, with more than a 5-fold enhancement of ligation obtained by increasing the pH from 7.5 to 9. In contrast, the ligation of the other acceptors did not show a significant pH dependence (supporting information, Table 1). Additionally, another product having increased electrophoretic mobility compared with the cleavage product, became readily apparent at high pH (indicated by the asterisk in Figure 5). This product most likely resulted from the displacement of the bound enzyme by water, affording the oligonucleotide GGCGCG-GAGACTTp, which has a 3′-phosphate group.[29] That this product actually contained a 3′-phosphate group was demonstrated by the decrease in electrophoretic mobility upon treat-

ment with T4 polynucleotide kinase,[30] which is known to have an associated 3′-phosphatase activity (data not shown).

Further characterization of differences in the ligation of the modified acceptors to the topoisomerase-DNA complex involved determination of the time course of the ligation reaction. The 5′-^{32}P end labeled complex was incubated with an excess of the individual acceptor oligonucleotides for various times, quenched by the addition of SDS, treated with proteinase K and analyzed by 20% denaturing PAGE. As shown in Figure 6, the modified acceptor oligonucleotides (**II**−**IV**) exhibited a significantly slower rate of ligation compared with the unmodified acceptor strand (**I**). Moreover, the observed efficiency of ligation at 60 min paralleled the rates of ligation of the individual acceptor oligonucleotides. It is interesting that the ligation reactions at 60 min in this experiment appear incomplete and the yields of ligation products were lower than those reflected in Table 1. This may well reflect the different amounts of acceptor oligonucleotides employed.

To establish the chemical nature of the newly formed bonds in the ligation products, these products were isolated from polyacrylamide gels and subjected to chemical and enzymatic degradation analysis. To determine the nature of the linkage formed by oligonucleotide **II**, an authentic synthetic product having the sequence shown in Figure 1B was synthesized by incorporation of the phosphoramidite monomer **2b** at cleavage site 1 (Figure 1). A synthetic oligonucleotide was also prepared containing the unmodified linkage at this site. The synthetic oligonucleotides were 5′-^{32}P end labeled and annealed to the noncleaved strand. The synthetic and enzymatically derived products were then incubated with exonuclease III, a nuclease that catalyzes the sequential removal of nucleotides from the ends of double-stranded DNA proceeding in a 3′ → 5′ direction. After incubation for 20 min at 37 °C, the reactions were quenched by the addition of EDTA and then analyzed on a 20% denaturing polyacrylamide gel. The synthetic and enzymatic ligation products containing the unmodified linkage at site 1 were completely degraded by exonuclease III (supporting information, Figure 6, lanes 3 and 6). In contrast, exonuclease III digestion of the ligation product putatively containing nucleoside **2a** at site 1, as well as the authentic synthetic standard containing **2a**, "stalled" at a specific site (arrow, lanes 4 and 5). Comparison of the degradation products with the Maxam-Gilbert G and G + A base-specific reactions revealed that exonuclease III digestion was strongly inhibited prior to the modified linkage (5′-^{32}pGGCGCGGAGACTTCH$_2$AGA-3′).

Consistent with the formation of a phosphorous-sulfur linkage, the ligation product afforded by the acceptor oligonucleotide **III** underwent oxidative cleavage when treated with 50 mM iodine in pyridine for 2 h at room temperature (Figure 7, cf lanes 3 and 4).[31] In contrast, the unmodified ligation product derived from **I** was refractory to cleavage with I_2 (Figure 7, cf. lanes 1 and 2).

The extreme lability of internucleosidic phosphoramidate linkages in dilute acid, relative to the phosphodiester linkage, provided a facile method to authenticate the linkage at site 1 of the ligation product formed by **IV**.[32] The putative phosphoramidate and phosphodiester-containing ligation products, formed by incubation of **IV** and **I**, respectively, were treated with 15% acetic acid for 12 h at room temperature. As shown in Figure 8, the product afforded by enzymatic ligation of **IV** underwent site-specific cleavage, yielding a single cleavage product (cf. lanes 2 and 3). The hydrolytic product had slightly increased mobility compared with synthetic 13-mer cleavage product (5′-^{32}pGGCGCGGAGACTT-3′) owing to the presence of a 3′-

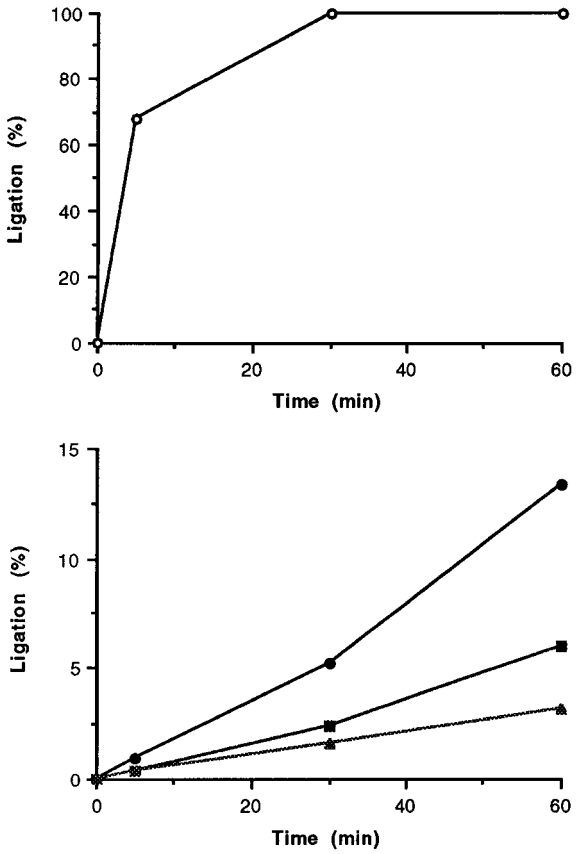


Figure 6. Time courses for the topoisomerase I-mediated ligation of the acceptor oligonucleotides (open circle, **I**; filled circle, **II**; filled square, **III**, filled triangle, **IV**).

Figure 7. Autoradiogram of a 20% denaturing polyacrylamide gel demonstrating the topoisomerase I-mediated formation of the bridging phosphorothioate linkage. Lane 1, I_2 treatment of the product resulting from ligation of **I** with the topoisomerase I−DNA covalent complex; lane 2, product resulting from ligation of **I** with the topoisomerase I−DNA covalent complex; lane 3, I_2 treatment of the product resulting from ligation of **III** with the topoisomerase I−DNA covalent complex; lane 4, product resulting from ligation of **III** with the topoisomerase I−DNA covalent complex.

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(32) Letsinger, R. L.; Wilkes, J. S.; Dumas, L. B. *Biochemistry* **1976**, *15*, 2810.

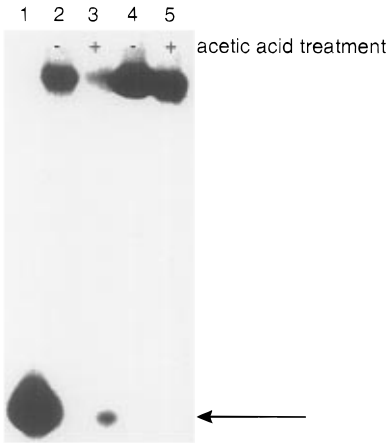

Figure 8. Autoradiogram of 20% denaturing polyacrylamide gel demonstrating the topoisomerase I-mediated formation of the phosphoramidate linkage. Lane 1, synthetic 13-mer cleavage product 5′^{32}pGGCGCGGAGACTT; lane 2, ligation product derived from **IV**; lane 3, ligation product derived from **IV** + acetic acid; lane 4, ligation product derived from **I**; lane 5, ligation product derived from **I** + acetic acid.

phosphate (cf. lanes 1 and 3). The cleavage product observed supports the presence of a phosphoramidate linkage at site 1.

Discussion

The catalytic cycle mediated by DNA topoisomerase I involves cleavage and ligation of the phosphodiester backbone of DNA via a transient DNA-enzyme covalent intermediate containing a phosphorotyrosine linkage. The equilibrium for the cleavage and ligation reactions that comprise the catalytic cycle lies strongly in the direction of the ligation reaction, affording a low steady-state concentration of covalent binary complexes.[5] The cleavage−ligation equilibrium can be altered by the camptothecins, which stabilize the covalent intermediate by inhibiting the ligation reaction.[33] *In vivo*, increasing the concentration of the topoisomerase I−DNA covalent complex facilitates chromosomal aberrations, sister chromatid exchange and illegitimate recombination.[3,34] This process can be modeled *in vitro* though the use of DNA duplexes containing a single discontinuity on the scissile strand in proximity to the preferred site of cleavage by topoisomerase I.[8,9] Site specific cleavage by the enzyme results in uncoupling of the cleavage and ligation reactions due to the instability of the short DNA duplex structure downstream from the site of cleavage (Figure 1A). The topoisomerase I trapped using such "suicide substrates" can undergo ligation with exogenously added DNA acceptors, affording structural transformations of the DNA; these have included insertions, deletions and mismatches.[8,9] We were, therefore, interested in determining whether topoisomerase I could catalyze additional alterations of DNA structure.

Christiansen et al.[29] have noted that nucleophilic species such as H_2O and glycerol, present at high concentrations in incubation mixtures containing topoisomerase I and DNA, could react with the electrophilic linkage formed between the active site tyrosine moiety of the enzyme and the phosphate ester of DNA. Accordingly, a nicked DNA substrate was utilized to uncouple the cleavage and ligation reactions of topoisomerase I, thereby allowing investigation of the nature of the nucleophiles capable of displacing the enzyme-linked tyrosine moiety from the DNA *when the nucleophiles were present as part of the acceptor oligonucleotide*. Enzyme-mediated ligation of the modified acceptors would afford a site-specific alteration of the ligated oligonucleotide at the site of ligation (Figure 1B). Acceptor oligonucleotides were synthesized containing 5′-thio and amino moieties; also prepared was an acceptor oligonucleotide with an additional methylene group at the 5′-terminus to permit investigation of spatial constraints for ligation.

Initial attempts utilizing a coupled cleavage−ligation assay suggested that topoisomerase I could mediate the joining of modified acceptor oligonucleotides, but product formation was limited by the availability of the enzyme−DNA intermediate. Therefore, anion exchange chromatography was utilized to obtain homogenous preparations of the covalent intermediate. This species was shown to be catalytically competent (Figure 3), thereby allowing investigation of the ligation reaction independent of the cleavage reaction. This approach has also been utilized by other workers to study DNA modifying enzymes; for example, Chen et al.[35] have isolated a stable covalent complex formed between DNA and the DNA (cytosine-5)-methyltransferase. For topoisomerase I, this method should provide access to large quantities of the enzyme−DNA intermediate required for physicochemical characterization of the formed binary complex. The results obtained here further suggest the general utility of this method for the isolation of protein−DNA complexes, e.g. for the site-specific prokaryotic integrases which have biochemical properties similar to topoisomerase I.[36,37]

Reaction of the purified DNA-topoisomerase I intermediate with acceptor oligonucleotides containing free 5′-CH_2OH, -SH, and -NH_2 moieties afforded DNA products containing modified internucleosidic bonds. The enzyme−DNA complex exhibited the greatest tolerance (29% yield) for acceptor oligonucleotide **II**, containing an additional methylene group at the 5′-terminus. This is consistent with the ability of topoisomerase I to facilitate the formation of insertions.[8,9] The acceptor oligonucleotides incorporating 5′-SH and -NH_2 groups both afforded significantly lower levels of ligation (13% and 4% conversion, respectively).

It was possible to increase the ligation yields obtained with acceptor oligonucleotide **IV** by increasing the pH of the incubation mixture. At pH 7.5, only 2.6% ligation was obtained, while at pH 9.0 12% of the substrate oligonucleotide was converted to full length product. As the pK_a of alkylamines is approximately 10, the pH effect most likely resulted from deprotonation of the amino moiety, thus increasing the nucleophilicity of this acceptor. Consistent with this interpretation, the incorporation of the other acceptors did not follow this trend as a function of pH. The latter observation is entirely consistent with the previous finding that the topoisomerase I-mediated ligation reaction of acceptor oligonucleotides containing a free 5′-OH group was independent of pH over the range 5.1−9.5.[29,38]

As noted above, Westergaard and coworkers have reported that the covalently bound topoisomerase I in the enzyme−DNA complex can be displaced by nucleophiles present in the reaction mixtures at high concentrations, including water and a variety of compounds with free hydroxyl groups, such as glycerol. The pH optima observed for these reactions were between 8.5 and 9.5.[29] Interestingly, mononucleosides and mononucleotides

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(37) While no definitive studies have been carried out to define the stability of the isolated covalent binary complex, experiments carried out during this study suggest that it can be maintained at low temperature at least for several hours. Significant loss of enzymatic competence was observed in one experiment after storage of the complex for 19 h at 4 °C.

(38) Stivers, J. T.; Shuman, S.; Mildvan, A. S. *Biochemistry* **1994**, *33*, 15449.

present at the same molar concentrations were unable to serve as substrates for ligation.[39]

Recently, the effect of a modified DNA linkage on the topoisomerase I-mediated cleavage reaction has been studied utilizing a DNA substrate containing a bridging phosphorothioate moiety.[40] Topoisomerase I was capable of cleaving the modified linkage to afford an oligonucleotide having a free 5′-SH moiety; the latter was not observed to undergo the reverse (i.e., ligation) reaction and was concluded to be incapable of doing so. This conclusion was based on the observed enhancement of cleavage of the substrate containing the phosphorothioate linkage, relative to the cleavage of the analogous substrate containing a phosphodiester linkage. The observed topoisomerase I cleavage represents the steady state population of covalent binary complexes. For the unmodified substrate, the covalent binary complex is present at low levels reflecting an equilibrium that favors the ligation reaction. For the substrate containing the phosphorothioate linkage, the increase in covalent binary complex formation was interpreted as the inability of the derived sulfhydryl-containing oligonucleotide to serve as a substrate for the reverse (i.e., ligation) reaction. It should be noted, however, that if the ligation reaction actually were infeasible, all of the phosphorothioate-containing substrate should eventually undergo cleavage. The fact that only partial conversion to cleaved product was actually observed implies either that insufficient time was provided to permit complete substrate cleavage, or else that there actually was an equilibrium between cleavage and ligation. In fact, the present results demonstrate that the 5′-SH functionality can displace the covalently bound enzyme to effect ligation, but at a decreased rate compared with the 5′-OH group normally present. This could well alter the cleavage−ligation equilibrium, consistent with the previously reported results.[40] Caution should be exercised when utilizing phosphorothioate-containing oligonucleotides as suicide substrates, if one is to conclude that the reaction is irreversible.

We have demonstrated previously that DNA topoisomerase I can promote the rearrangment of DNA structure; catalysis of nucleotide insertions and deletions was documented for a few types of systems.[8] In the present study, we have extended these findings by exploring the range of nucleophiles capable of reacting with the activated enzyme−DNA complex. In particular, it is known that phenoxides undergo phosphoryl transfer reactions with facility,[41] a type of transformation that nature apparently exploits in the use of tyrosine as the active site nucleophile for reaction with the phosphate ester backbone of DNA. The ability of S and N nucleophiles to promote the loss of this tyrosine phenoxide moiety is of fundamental interest from a chemical perspective, as it reflects the extent to which a biochemical transformation can be influenced by simple alteration of key chemical parameters. In fact, the pH-dependent extent of ligation obtained using acceptor oligonucleotide **IV** argues that the facility of the ligation reaction is explicable in terms of simple chemical principles.

In similar fashion, the lack of effect of Mg^{2+} or Mn^{2+} on the ligation reaction carried out in the presence of acceptor oligonucleotide **III** has important implications for the role of metal ions in DNA strand scission and ligation by topoisomerase I. The ability of oligonucleotide **II** to undergo ligation with quite reasonable efficiency reflects considerable flexibility in the spatial requirements for religation, a parameter that may also operate during topoisomerase I-mediated nucleotide deletion and insertion reactions.[8]

Finally, definition of the ease with which specific nucleophiles can react with the electrophilic topoisomerase I−DNA binary complex should facilitate the exploration of novel strategies for intercepting the activated enzyme−DNA binary complex.

Experimental Section

General Methods. Elemental analyses were carried out by Atlantic Microlab, Inc., Norcross, GA. Melting points were taken on a Thomas Hoover apparatus and are not corrected. Optical rotations were determined on a Perkin-Elmer Model 141 polarimeter. ^{1}H NMR and ^{13}C NMR spectra were recorded on a General Electric QE-300 spectrophotometer. Chemical shifts values are expressed relative to added tetramethylsilane. Experiments requiring anhydrous conditions were performed under an argon atmosphere. Solvents were J. T. Baker p. a. and were used without further purification unless noted. THF and diethyl ether were distilled from potassium-benzophenone. Thin layer chromatography (TLC) was carried out on Merck silica gel F_{245} pre-coated plates; spots were visualized by dipping the plates in a Ce-Mo staining reagent. Column chromatography employed Fluka silica gel 60, mesh size 230−400.

T4 polynucleotide kinase and proteinase K were purchased from United States Biochemical; exonuclease III was from Gibco BRL. β-Cyanoethylphosphoramidites, activator solution and solid support were obtained from Cruachem Inc. Nensorb prep nucleic acid purification cartridges were from DuPont-New England Nuclear; [γ-^{32}P]-ATP (7000 Ci/mmol) was obtained from ICN Biochemicals. Scintillation counting was performed on a Beckman LS-100C instrument using Beckman Ready Safe scintillation fluid. The Mono Q (HR 5/5) column was from Pharmacia and FPLC was performed on a Pharmacia system.

Polyacrylamide gel electrophoresis was carried out on 20% gels [19% (w/v) acrylamide, 1% (w/v) *N*,*N*-methylenebisacrylamide, 8 M urea] in 90 mM Tris-borate buffer, pH 8.3, containing 5 mM EDTA. Polyacrylamide gel loading solution contained 10 M urea, 1.5 mM EDTA, 0.05% (w/v) xylene cyanol and 0.05% (w/v) bromphenol blue. Gels were visualized by autoradiography at −80 °C with Kodak XAR-2 film and quantified utilizing the Molecular Dynamics 400E Phosphorimager using ImageQuant version 3.2 software. Sequencing analysis was performed using a modification[42] of the traditional Maxam-Gilbert method[43] for short, single-stranded deoxyoligonucleotides. Distilled, deionized water from a Milli-Q system was used for all aqueous manipulations.

Synthesis of Nucleosides. 3-*O*-Benzyl-1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose (6). To a stirred solution containing 3.0 g (11.5 mmol) of diacetone allofuranose (**5**) and 660 mg (16.5 mmol) of sodium hydride (60% dispersion in mineral oil) in 50 mL of DMF was added 1.40 mL (11.7 mmol) of benzyl bromide. The combined solution was stirred at room temperature for 1 h, then cooled to 0 °C. The reaction mixture was partitioned between 50 mL of saturated $NaHCO_3$ and 200 mL of ethyl acetate. The organic phase was washed with 50 mL of brine and dried over $MgSO_4$. The solution was concentrated under diminished pressure to yield a crude product which was purified by flash chromatography on a silica gel column (25 cm × 3 cm). Elution with 3:7 ethyl acetate−hexanes afforded **6** as a viscous, colorless oil that solidified at room temperature: yield 4.03 g (quantitative); mp 64 °C; silica gel TLC R_f 0.61 (3:7 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 1.35 (s, 3 H), 1.36 (s, 3 H), 1.38 (s, 3 H), 1.58 (s, 3 H), 3.87 (dd, 1 H, J = 4.5, 8.5 Hz), 3.93−4.04 (m, 2 H), 4.14 (dd, 1 H, J = 3, 8.5 Hz), 4.35 (ddd, 1 H, J = 3, 7, 10 Hz), 4.57 (d, 1 H, J = 8 Hz), 4.59 (d, 1 H, J = 11.5 Hz), 4.77 (d, 1 H, J = 11.5 Hz), 5.74 (d, 1 H, J = 3.5 Hz) and 7.30−7.41 (m, 5 H); ^{13}C NMR ($CDCl_3$) δ 25.5, 26.6, 27.0, 27.3, 65.6, 72.6, 75.2, 77.9, 78.2, 78.5, 104.3, 110.0, 113.3, 128.4, 128.6, 128.8 and 137.9. Anal. Calcd for $C_{19}H_{26}O_6$: C, 65.12; H, 7.48. Found: C, 65.42; H, 7.56.

3-*O*-Benzyl-1,2:5,6-tetra-*O*-acetyl-β-D-allofuranose (7). To a stirred solution containing 4.03 g (11.5 mmol) of diacetonide **6** in 120 mL of acetic acid was added 4.8 mL (69 mmol) of acetic anhydride, followed

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(42) (a) Jay, E.; Seth, A. K.; Rommens, J.; Sood, A.; Jay, G. *Nucleic Acids Res.* **1982**, *10*, 6319. (b) Banaszuk, A. M.; Deugau, K. V.; Sherwood, J.; Michalak, M.; Glick, B. R. *Anal. Biochem*. **1983**, *128*, 281.

by 200 mg (1.15 mmol) of *p*-toluenesulfonic acid monohydrate. The reaction mixture was heated at reflux for 1 h and then the solvent was coevaporated with two 100-mL portions of toluene under diminished pressure. The residue was dissolved in 250 mL of ethyl acetate, washed with 100 mL of saturated $NaHCO_3$, and then dried over $MgSO_4$. The solution was concentrated under diminished pressure to afford a residue, which was purified by flash chromatography on a silica gel column (20 cm × 4 cm). Elution with 3:7 ethyl acetate−hexanes afforded **7** as a colorless solid: yield 4.90 g (97%) as a 9:1 mixture of anomers. The diastereomeric ratio was determined according to the signals of anomeric protons in the crude product. The major anomer was isolated as colorless microcrystals from ethyl acetate−hexanes; mp 86 °C; silica gel TLC R_f 0.50 (3:7 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 2.03 (s, 3 H), 2.04 (s, 3 H), 2.09 (s, 3 H), 2.12 (s, 3 H), 4.05 (dd, 1 H, J = 6.5, 12 Hz), 4.19 (dd = t, 1 H, J = 7.5 Hz), 4.30 (dd, 1 H, J = 5, 8 Hz), 4.36 (dd, 1 H, J = 3, 9.5 Hz), 4.43 (d, 1 H, J = 11 Hz), 4.58 (d, 1 H, J = 11 Hz), 5.23−5.28 (m, 1 H), 5.30 (d, 1 H, J = 4 Hz), 6.14 (s, 1 H) and 7.28−7.35 (m, 5 H); ^{13}C NMR ($CDCl_3$) δ 21.10, 21.12, 21.3, 21.4, 63.0, 71.8, 73.8, 78.7, 80.3, 98.8, 128.0, 128.5, 128.6, 128.9, 137.2, 169.2, 170.1, 170.4 and 170.9. Anal. Calcd for $C_{21}H_{26}O_{10}$: C, 57.53; H, 5.98. Found: C, 57.47; H, 5.85.

***N*^{6}-Benzoyl-9-[(2*R*,3*R*,4*R*,5*R*)-3-(acetoxy)-4-(benzoxy)-5-[(1*R*)-1,2-(diacetoxy)ethyl]tetrahydrofuran-2-yl]adenine (8).** Trimethylsilyl trifluoromethanesulfonate (540 μL, 2.70 mmol) was added to a stirred solution containing 4.90 g (11.2 mmol) of tetraacetate **7** and 4.30 g (11.2 mmol) of bis-TMS-*N*^{6}-benzoyladenine in 200 mL of CH_3CN at room temperature. The combined solution was heated at 55 °C for 12 h. After cooling to 0 °C, the reaction was quenched by the addition of 50 mL of saturated $NaHCO_3$ followed by the addition of 250 mL of ethyl acetate. The organic layer was separated, washed successively with two 100-mL portions of saturated $NaHCO_3$ and two 100-mL portions of brine, then dried over $MgSO_4$. The solution was concentrated under diminished pressure to afford a residue which was purified by flash chromatography on a silica gel column (25 cm × 4 cm). Elution with 9:1 CH_2Cl_2−methanol afforded nucleoside **8** as a colorless foam: yield 4.91 g (71%); mp 69 °C; silica gel TLC R_f 0.70 (9:1 CH_2Cl_2−methanol); ^{1}H NMR ($CDCl_3$) δ 1.97 (s, 3 H), 2.04 (s, 3 H), 2.11 (s, 3 H), 4.05 (dd, 1 H, J = 6, 12 Hz), 4.32 (t, 1 H, J = 6 Hz), 4.38 (dd, 1 H, J = 3.5, 12 Hz), 4.62 (d, 1 H, J = 12.5 Hz), 4.65 (d, 1 H, J = 12.5 Hz), 4.82 (t, 1 H, J = 5.5 Hz), 5.43−5.48 (m, 1 H), 5.95 (t, 1 H, J = 4 Hz), 6.09 (d, 1 H, J = 4 Hz), 7.30−7.40 (m, 5 H), 7.50−7.65 (m, 3 H), 8.01−8.04 (m, 3 H), 8.02 (s, 1 H) and 8.91 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 21.0, 21.1, 21.2, 62.5, 70.6, 73.6, 76.7, 81.2, 88.1, 124.4, 128.3, 128.6, 128.7, 128.9, 129.2, 133.2, 133.9, 137.2, 142.8, 150.3, 151.8, 153.1, 165.2, 170.1, 170.2 and 170.8; mass spectrum (FABMS) *m/z* 618.218 (M + H)$^+$ ($C_{31}H_{32}N_5O_9$ requires 618.220).

***N*^{6}-Benzoyl-9-[(2*R*,3*R*,4*R*,5*R*)-4-(benzoxy)-5-[(1*R*)-1,2-(dihydroxy)ethyl]-3-(hydroxy)tetrahydrofuran-2-yl]adenine (9).** A solution of 4.91 g (7.96 mmol) of nucleoside analogue **8** in 18 mL of 2:1 ethanol-pyridine was treated with 6 mL of 2 N NaOH in 6 mL of ethanol at room temperature. The combined solution was stirred at room temperature for 10 min, then cooled to 0 °C. The solution was adjusted to pH 7 with 1 N HCl, then extracted with 250 mL of ethyl acetate. The organic layer was washed successively with 100 mL of saturated $NaHCO_3$ and 100 mL of brine, then dried over $MgSO_4$. The solution was concentrated under diminished pressure to afford a residue, which was purified by flash chromatography on a silica gel column (20 cm × 3 cm). Elution with 9:1 CH_2Cl_2−methanol afforded nucleoside **9** as a white foam: yield 3.36 g (86%); mp 110 °C; silica gel TLC R_f 0.50 (9:1 CH_2Cl_2−methanol); ^{1}H NMR (CD_3OD) δ 2.54 (d, 2 H, J = 6 Hz), 3.92−3.97 (m, 1 H), 4.23 (d, 1 H, J = 5 Hz), 4.30 (s, 1 H), 4.63 (d, 1 H, J = 7.5 Hz), 4.80 (d, 1 H, J = 7.5 Hz), 4.91 (d, 1 H, J = 6 Hz), 6.07 (d, 1 H, J = 7 Hz), 7.33 (m, 8 H), 7.97 (d, 2 H, J = 7 Hz), 8.43 (s, 1 H) and 8.54 (s, 1 H); ^{13}C NMR (CD_3OD) δ 62.9, 72.2, 72.4, 74.2, 77.3, 85.5, 89.6, 124.2, 127.7, 128.5, 128.7, 132.9, 133.7, 138.3, 143.9, 150.2, 151.7, 152.0 and 166.7; mass spectrum (FABMS) *m/z* 492.1891 (M + H)$^+$ ($C_{25}H_{26}N_5O_6$ requires 492.188).

***N*^{6}-Benzoyl-9-[(2*R*,3*R*,4*R*,5*R*)-4-(benzoxy)-5-[(1*R*)-2-(*tert*-butyldiphenylsilyloxy)-1-(hydroxy)ethyl]-3-(hydroxy)tetrahydrofuran-2-yl] adenine (10).** *tert*-Butyldiphenylsilyl chloride (790 μL, 3.05 mmol) was added dropwise to a stirred solution of 1.50 g (3.05 mmol) of triol **9** and 829 mg (12.2 mmol) of imidazole in 80 mL of CH_2Cl_2. The combined solution was stirred at room temperature for 1 h, then diluted with 150 mL of ethyl acetate. The reaction mixture was washed successively with 50 mL of saturated $NaHCO_3$ and 50 mL of brine, then dried over $MgSO_4$. The solution was concentrated under diminished pressure to afford a residue which was purified by flash chromatography on a silica gel column (20 cm × 3 cm). Elution with 9:1 CH_2Cl_2−methanol afforded **10** as a colorless foam: yield 2.17 g (97%); mp 112 °C; silica gel TLC R_f 0.70 (9:1 CH_2Cl_2−methanol); ^{1}H NMR (CD_3OD) δ 0.99 (s, 9 H), 3.59 (dd, 1 H, J = 7, 10.5 Hz), 3.70 (dd, 1 H, J = 5, 10 Hz), 4.01−4.06 (m, 1 H), 4.21 (d, 1 H, J = 5 Hz), 4.50 (s, 1 H), 4.62 (d, 1 H, J = 12 Hz), 4.67 (d, 1 H, J = 12 Hz), 4.92 (t, 1 H, J = 6 Hz), 6.09 (d, 1 H, J = 7 Hz) 7.12−7.71 (m, 18 H), 7.98 (d, 2 H, J = 7.5 Hz), 8.42 (s, 1 H) and 8.51 (s, 1 H); ^{13}C NMR (CD_3OD) δ 19.1, 26.6, 64.9, 72.1, 72.2, 74.4, 77.0, 85.5, 89.6, 124.3, 127.6, 127.9, 127.99, 128.5, 128.7, 129.5, 130.0, 132.2, 132.9, 133.3, 133.4, 133.8, 135.0, 135.7, 138.2, 143.9, 150.2, 151.7, 151.9 and 166.7; mass spectrum (FABMS) *m/z* 730.308 (M + H)$^+$ ($C_{41}H_{44}N_5O_6Si$ requires 730.306).

***N*^{6}-Benzoyl-9-[(2*R*,3*R*,4*R*,5*R*)-4-(benzoxy)-5-[(1*R*)-2-[(*tert*-butyldiphenylsilyl)oxy]-1-[(imidazolethiocarbonyl)oxy]ethyl]-3-[(imidazolethiocarbonyl)oxy]tetrahydrofuran-2-yl]adenine (11).** Thiocarbonyldiimidazole (2.0 g, 10.0 mmol) was added quickly to a stirred solution containing 2.17 g (2.97 mmol) of **10** in 75 mL of THF. The reaction mixture was stirred at 65 °C for 12 h in the dark, then the solution was concentrated under diminished pressure. The resulting residue was dissolved in 200 mL of 5:1 ethyl acetate-CH_2Cl_2. The solution was washed successively with cold 1 N HCl (50 mL), saturated $NaHCO_3$ (75 mL) and brine (75 mL), then dried over $MgSO_4$. The solution was concentrated under diminished pressure to afford a residue which was purified by flash chromatography on a silica gel column (25 cm × 3 cm). Elution with ethyl acetate followed by 9:1 CH_2Cl_2−methanol afforded **11** as a colorless powder: yield 2.52 g (90%); silica gel TLC R_f 0.60 (9:1 CH_2Cl_2−methanol); ^{1}H NMR ($CDCl_3$) δ 1.04 (s, 9 H), 3.94 (dd, 1 H, J = 5.5, 10.5 Hz), 4.00 (dd, 1 H, J = 5.5, 10.5 Hz), 4.50 (d, 1 H, J = 11.5 Hz), 4.56 (d, 1 H, J = 11.5 Hz), 4.74 (t, 1 H, J = 5.5 Hz), 5.21 (t, 1 H, J = 6 Hz), 6.21 (d, 1 H, J = 4 Hz), 6.24 (t, 1 H, J = 6 Hz), 6.45 (dd, 1 H, J = 4, 5 Hz), 7.02−7.63 (m, 22 H), 7.98 (s, 1 H), 8.01 (m, 2 H), 8.26 (s, 1 H), 8.33 (s, 1 H), 8.50 (s, 1 H) and 8.80 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 18.6, 26.3, 61.0, 72.9, 75.0, 79.9, 80.1, 81.0, 86.0, 117.4, 123.9, 127.3, 127.7, 128.0, 128.1, 129.5, 130.3, 130.7, 131.9, 132.1, 133.1, 134.9, 135.1, 136.6, 136.8, 142.6, 149.9, 150.8, 151.9, 165.0, 182.0 and 182.7. Anal. Calcd for $C_{49}H_{47}O_9N_9SiS_2$: C, 61.93; H, 4.99. Found: C, 61.71; H, 5.18.

***N*^{6}-Benzoyl-9-[(2*R*,4*S*,5*R*)-4-(benzoxy)-5-[(*tert*-butyldiphenylsilyloxy)ethyl]tetrahydrofuran-2-yl]adenine (12).** To a stirred solution containing 804 μL (2.98 mmol) of Bu_3SnH and 25 mg (0.15 mmol) of azobisisobutyronitrile (AIBN) in 50 mL of toluene at 75 °C was added dropwise a solution of 951 mg (1.00 mmol) of **11** in 30 mL of toluene. The combined solution was stirred at 75 °C for 90 min and then concentrated under diminished pressure to afford a residue, which was purified by flash chromatography on a silica gel column (20 cm × 2 cm). Elution with ethyl acetate afforded **12** as a colorless foam: yield 560 mg (80%); mp 58−61 °C; silica gel TLC R_f 0.40 (3:1 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 1.05 (s, 9 H), 1.91−1.97 (m, 2 H), 2.63 (ddd, 1 H, J = 3, 6, 9 Hz), 2.85 (dt, 1 H, J = 6, 13.5 Hz), 3.78 (t, 2 H, J = 6 Hz), 4.21−4.25 (m, 1 H), 4.46 (m, 1 H), 4.57 (d, 1 H, J = 10.5 Hz), 4.68 (d, 1 H, J = 10.5 Hz), 6.33 (t, 1 H, J = 6.5 Hz), 7.50 (m, 18 H), 7.99 (m, 2 H), 8.03 (s, 1 H), 8.77 (s, 1 H) and 8.91 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 19.6, 27.4, 37.5, 61.0, 66.2, 71.8, 82.2, 82.5, 85.4, 124.8, 127.1, 128.1, 128.2, 128.3, 128.5, 128.9, 130.2, 132.9, 134.1, 135.9, 138.1, 142.4, 150.4, 152.1, 152.6 and 165.9; mass spectrum (FABMS) *m/z* 698.315 (M + H)$^+$ ($C_{41}H_{44}N_5O_4Si$ requires 698.316).

***N*^{6}-Benzoyl-9-[(2*R*,4*S*,5*R*)-4-(benzoxy)-5-(2-hydroxyethyl)tetrahydrofuran-2-yl]adenine (13).** To a solution containing 512 mg (0.73 mmol) of **12** in 25 mL of THF was added 800 μL (0.80 mmol) of a

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1M solution of tetrabutylammonium fluoride in THF. The reaction mixture was stirred at room temperature for 5 h. The solution was concentrated under diminished pressure to afford a residue, which was purified by flash chromatography on a silica gel column (25 cm × 3 cm). Elution with ethyl acetate, followed by 9:1 CH_2Cl_2−methanol, afforded nucleoside analogue **13** as a colorless foam: yield 254 mg (76%); mp 115−117 °C; silica gel TLC R_f 0.70 (9:1 CH_2Cl_2−methanol); ^{1}H NMR ($CDCl_3$) δ 1.96−2.03 (m, 2 H), 2.42 (s, 1 H), 2.59 (ddd, 1 H, J = 3.5, 6.5, 13.5 Hz), 2.91−2.99 (m, 1 H), 3.72−3.83 (m, 2 H), 4.29−4.37 (m, 2 H), 4.58 (d, 1 H, J = 11.5 Hz), 4.62 (d, 1 H, J = 11.5 Hz), 6.36 (t, 1 H, J = 6.5 Hz), 7.29−7.37 (m, 5 H), 7.51−7.63 (m, 3 H), 8.01 (d, 2 H, J = 7 Hz), 8.10 (s, 1 H), 8.77 (s, 1 H) and 8.99 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 36.7, 37.4, 59.5, 72.0, 82.1, 83.2, 85.3, 124.1, 128.1, 128.2, 128.3, 128.4, 128.9, 129.0, 133.0, 134.0, 137.8, 142.3, 150.1, 151.7, 152.7 and 165.7; mass spectrum (FABMS) *m/z* 458.183 $(M + H)^+$ ($C_{25}H_{26}N_5O_4$ requires 458.183).

***N*^{6}-Benzoyl-9-[(2*R*,4*S*,5*R*)-4-(hydroxy)-5-(2-hydroxyethyl)tetrahydrofuran-2-yl]adenine (14).** A solution containing 80 mg (0.17 mmol) of nucleoside **13** and freshly prepared palladium black (prepared[18] from 420 mg of $PdCl_2$) in 17 mL of dry ethanol was stirred under 1 atm of H_2 at 45 °C for 9 h. The solution was decanted and the catalyst was washed with 20 mL of ethanol. The combined solution was concentrated under diminished pressure to afford a residue that was purified by flash chromatography on a silica gel column (15 cm × 3 cm). Elution with 9:1 CH_2Cl_2−methanol afforded **14** as a colorless powder: yield 62 mg (83%); mp 142−145 °C; silica gel TLC R_f 0.40 (9:1 CH_2Cl_2-methanol); ^{1}H NMR (CD_3OD) δ 1.91 (m, 2 H), 2.48 (ddd, 1 H, J = 5, 6.5, 11.5 Hz), 2.94 (d, t, 1 H, J = 6.5, 13 Hz), 3.61−3.70 (m, 2 H), 4.05 (m, 1 H), 4.47 (m, 1 H), 6.48 (t, 1 H, J = 6.5 Hz), 7.51−7.65 (m, 3 H), 8.06 (d, 2 H, J = 7 Hz), 8.50 (s, 1 H) and 8.69 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 36.5, 39.2, 58.8, 74.5, 84.7, 124.4, 128.4, 128.5, 128.7, 132.8, 133.9, 143.4, 150.0, 152.1 and 167.1; mass spectrum (FABMS) *m/z* 370.149 $(M + H)^+$ ($C_{18}H_{20}N_5O_4$ requires 370.151).

***N*^{6}-Benzoyl-9-[(2*R*,4*S*,5*R*)-5-[2-(4,4′-dimethoxytriphenylmethoxy)ethyl]-4-(hydroxy)tetrahydrofuran-2-yl]adenine (2a).** To a solution containing 35 mg (0.095 mmol) of diol **14** in 1 mL of pyridine at 0 °C was added slowly 35.8 mg (0.10 mmol) of dimethoxytrityl chloride in 0.5 mL of pyridine. The combined solution was stirred at 0 °C for 1 h, then extracted with a mixture of saturated $NaHCO_3$ solution (25 mL) and ethyl acetate (100 mL). The organic layer was washed with 25 mL of brine, then dried over $MgSO_4$. The dried solution was concentrated under diminished pressure to afford a residue which was purified by flash chromatography on a silica gel column (20 cm × 3 cm). Elution with 9:1 CH_2Cl_2−methanol afforded **2a** as a colorless powder: yield 52 mg (81%); mp 113−115 °C; silica gel TLC R_f 0.50 (9:1 CH_2Cl_2−methanol); ^{1}H NMR ($CDCl_3$) δ 1.97−2.08 (m, 2 H), 2.58 (dt, 1 H, J = 7, 13 Hz), 2.89 (ddd, 1 H, J = 4, 7, 11.5 Hz), 3.15 (dd, 1 H, J = 4, 9 Hz), 3.21 (d, 1 H, J = 2.5 Hz), 3.41−3.47 (m, 1 H), 3.78 (s, 3 H), 3.79 (s, 3 H), 3.91−3.97 (m, 1 H), 4.54−4.60 (m, 1 H), 6.48 (dd, 1 H, J = 4, 7 Hz), 6.82−6.86 (m, 4 H), 7.19−7.64 (m, 12 H), 8.01−8.04 (m, 2 H), 8.10 (s, 1 H), 8.80 (s, 1 H) and 8.94 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 32.0, 34.4, 40.1, 55.6, 61.1, 74.7, 84.5, 86.2, 87.4, 113.7, 124.0, 127.3, 128.3, 128.4, 129.0, 130.2, 130.3, 133.1, 136.0, 136.3, 141.7, 144.9, 153.0, 158.0, and 166.2; mass spectrum (FABMS) *m/z* 672.284 $(M + H)^+$ ($C_{39}H_{38}N_5O_6$ requires 672.282).

***N*^{6}-Benzoyl-9-[(2*R*,4*S*,5*S*)-4-[(2-cyanoethyl *N*,*N*-diisopropylphosphoramidityl)oxy]-5-[2-(4,4′-dimethoxytriphenylmethoxy)ethyl]-tetrahydrofuran-2-yl]adenine (2b).** To a stirred solution containing 60 mg (0.089 mmol) of **2a** in 4 mL of CH_2Cl_2 was added successively at room temperature 40 μL (0.23 mmol) of *N*,*N*-diisopropylethylamine and 19.8 μL (0.089 mmol) of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite. The reaction mixture was stirred at room temperature for 1 h, then diluted with 50 mL of ethyl acetate. The solution was washed with 15 mL of saturated $NaHCO_3$, dried over $MgSO_4$, and then concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 cm × 3 cm). Elution with 45:45:10 CH_2Cl_2−ethyl acetate−Et_3N afforded monomer **2b** as a colorless foam: yield 61 mg (77%) of a 1:1 mixture of diastereomers; silica gel TLC R_f 0.70 (45:45:10 CH_2Cl_2−ethyl acetate−Et_3N); ^{1}H NMR ($CDCl_3$) δ 1.21−1.25 (m, 12 H), 1.87−2.11 (m, 2 H), 2.53−2.70 (m, 3 H), 2.85−2.93 (m, 1 H), 3.18−3.24 (m, 2 H), 3.52−3.71 (m, 3.5 H), 3.77 (s, 6 H), 3.80−3.88 (m, 0.5 H), 4.31−4.37 (m, 1 H), 4.59−4.62 (m, 1 H), 6.37 (t, 1 H, J = 6.5 Hz), 6.79 (dd, 4 H, J = 3, 9 Hz), 7.16−7.64 (m, 12 H), 7.97−8.03 (m, 3 H), 8.77 (s, 0.5 H), 8.79 (s, 0.5 H) and 8.96 (s, 1 H); mass spectrum (FABMS) *m/z* 872.390 $(M + H)^+$ ($C_{48}H_{55}N_7O_7P$ requires 872.390).

***N*^{6}-Benzoyl-3′-*O*-benzyladenosine (15).** Sodium periodate (120 mg, 0.56 mmol) was added to a stirred solution containing 200 mg (0.41 mmol) of nucleoside analogue **9** in 10 mL of 9:1 THF−H_2O. The suspension was stirred vigorously at room temperature for 2 h, then concentrated under diminished pressure. The residue was dissolved in 25 mL of methanol and filtered. To the filtrate was added 200 mg (5.28 mmol) of $NaBH_4$ at 0 °C. The solution was stirred at room temperature for 30 min and then cooled to 0 °C. The solution was adjusted to pH 7 with 1 N HCl, then extracted with 150 mL of ethyl acetate. The organic layer was separated, washed successively with 50 mL of saturated $NaHCO_3$ and 50 mL brine, then dried over $MgSO_4$. The dried solution was concentrated under diminished pressure to afford a residue which was purified by flash chromatography on a silica gel column (15 cm × 3 cm). Elution with 9:1 CH_2Cl_2−methanol afforded diol **15** as a colorless foam: yield 146 mg (83%); mp 200 °C; silica gel TLC R_f 0.60 (9:1 CH_2Cl_2−methanol); ^{1}H NMR (CD_3OD) δ 3.58 (dd, 1 H, J = 1.5, 13 Hz), 3.88 (dd, 1 H, J = 1.5, 13 Hz), 4.22 (dd, 1 H, J = 1.5, 5 Hz), 4.30 (d, 1 H, J = 1.5 Hz), 4.65 (d, 1 H, J = 11.5 Hz), 4.71 (d, 1 H, J = 11.5 Hz), 4.83 (dd, 1 H, J = 6, 7 Hz), 5.88 (d, 1 H, J = 7 Hz), 7.30 (m, 5 H), 7.43−7.57 (m, 3 H), 8.00 (m, 2 H), 8.15 (s, 1 H) and 8.67 (s, 1 H). Anal. Calcd for $C_{24}H_{23}O_5N_5$: C, 62.46; H, 5.02. Found: C, 62.09; H, 4.98.

***N*^{6}-Benzoyladenosine (16).** A suspension of palladium black (freshly prepared[18] from 500 mg of $PdCl_2$) and 110 mg (0.24 mmol) of nucleoside **15** in 10 mL of ethanol was stirred under 1 atm of H_2 at 45 °C for 9 h. The solution was removed by decatenation and the catalyst was washed with 20 mL of ethanol. The combined solution was concentrated under diminished pressure to afford a residue which was purified by flash chromatography on a silica gel column (20 cm × 3 cm). Elution with 9:1 CH_2Cl_2−methanol afforded *N*^{6}-benzoyladenosine (**16**) as a colorless powder: yield 70 mg (68%); silica gel TLC R_f 0.50 (9:1 CH_2Cl_2−methanol); $[\alpha]_D$ −48.0° (*c* 0.5, MeOH) (authentic sample $[\alpha]_D$ −47.8° (*c* 0.5, MeOH)); ^{1}H NMR (CD_3OD) δ 3.75 (dd,1 H, J = 3, 12 Hz), 3.88 (dd, 1 H, J = 3, 13 Hz), 4.15−4.17 (m, 1 H), 4.34 (dd, 1 H, J = 3, 3.5 Hz), 4.73 (t, 1 H, J = 5.5 Hz), 6.12 (d, 1 H, J = 6 Hz), 7.52−7.65 (m, 3 H), 8.05−8.07 (m, 2 H), 8.65 (s, 1 H) and 8.69 (s, 1 H).

5′-[(Acetyl)thio]-2′,5′-dideoxyadenosine (18). A solution containing 1.05 g (3.89 mmol) of 2′-deoxyadenosine (**17**) and 1.3 g (4.95 mmol) of Ph_3P in 45 mL of THF was treated successively with 779 μL (4.05 mmol) of diethyl azodicarboxylate and 353 μL (4.95 mmol) of thiolacetic acid. The reaction mixture was stirred at room temperature for 1 h and then concentrated under diminished pressure to afford a residue. The residue was purified by flash chromatography on a silica gel column (25 cm × 3 cm). Elution with 9:1 CH_2Cl_2−methanol afforded **18** as a colorless foam: yield 1.06 g (88%); mp 74−75 °C; silica gel TLC R_f 0.30 (9:1 CH_2Cl_2−methanol); ^{1}H NMR (CD_3OD) δ 2.38 (s, 3 H), 2.50−2.60 (m, 1 H), 2.88−2.96 (m, 1 H), 3.27 (dd, 1 H, J = 6, 12.5 Hz), 3.38 (dd, 1 H, J = 6, 12.5 Hz), 4.10−4.18 (m, 1 H), 4.45−4.55 (m, 1 H), 5.80 (br s, 2 H), 6.40 (t, 1 H, J = 6 Hz), 8.02 (s, 1 H) and 8.35 (s, 1 H); ^{13}C NMR (CD_3OD) δ 29.4, 31.4, 39.0, 73.6, 84.8, 86.1, 119.6, 140.1, 149.4, 152.8, 156.3 and 195.6; mass spectrum (FABMS) *m/z* 310.098 $(M + H)^+$ ($C_{12}H_{16}N_5O_3S$ requires 310.098).

5′-[(Acetyl)thio]-2′,5′-dideoxy-3′-*O*,*N*^{6},*N*^{6}-tribenzoyl)adenosine(19). To a stirred solution containing 800 mg (2.58 mmol) of thioester **18** in 15 mL of pyridine was added 1.19 mL (10.2 mmol) of benzoyl chloride at 0 °C. The combined solution was stirred at room temperature for 12 h, then diluted with 200 mL of ethyl acetate. The reaction mixture was washed successively with 100 mL of saturated $NaHCO_3$ and 100 mL of brine, then dried over $MgSO_4$. The dried solution was concentrated under diminished pressure to afford a residue which was purified by flash chromatography on a silica gel column (20 cm × 4 cm). Elution with 2:1 ethyl acetate−hexanes afforded **19** as a colorless foam: yield 1.48 g (92%); mp 97−101 °C; silica gel TLC R_f 0.65 (2:1 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 2.36 (s, 3 H), 2.74 (dd, 1 H, J = 2, 6 Hz), 3.14−3.24 (m, 1 H), 3.39 (dd, 1 H, J = 6, 12 Hz), 3.47 (dd, 1 H, J = 6, 14 Hz), 4.45−4.50 (m, 1 H), 5.55−5.58 (m, 1 H), 6.51 (dd, 1 H, J = 6, 8 Hz), 7.34−7.64 (m, 9 H), 7.86 (d, 4 H, J

= 7 Hz), 8.07 (d, 2 H, J = 7 Hz), 8.29 (s, 1 H) and 8.66 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 30.9, 31.8, 76.9, 84.0, 85.3, 128.4, 129.0, 129.1, 129.4, 129.8, 130.1, 133.4, 134.0, 144.0, 152.0, 152.4, 153.0, 166.0, 172.7 and 194.9; Anal. Calcd for $C_{33}H_{27}O_6N_5S$: C, 63.75; H, 4.38. Found: C, 63.61; H, 4.48.

***N*^{6}-Benzoyl-2′,5′-dideoxy-5′-thioadenosine (20).** A solution containing 900 mg (1.45 mmol) of nucleoside **19** in 10 mL of 2:1 EtOH-pyridine was treated under argon with 2.17 mL of 2 N NaOH and 2 mL of EtOH. The solution was degassed by stirring under vacuum for 2 min, then stirred for an additional 5 min at room temperature under argon. The solution was adjusted to pH 7 with 1 N HCl and extracted with a mixture of saturated $NaHCO_3$ solution (50 mL) and ethyl acetate (250 mL). The organic layer was dried over $MgSO_4$. The solvent was concentrated under diminished pressure to afford a residue, which was purified by flash chromatography on a silica gel column (25 cm × 3 cm). Elution with 9:1 CH_2Cl_2−methanol afforded **20** as a colorless foam: yield 484 mg (90%); mp 87−90 °C; silica gel TLC R_f 0.40 (9:1 CH_2Cl_2−methanol); ^{1}H NMR ($CDCl_3$) δ 1.60 (t, 1 H, J = 7.5 Hz), 2.40 (br s, 1 H), 2.52−2.62 (m, 1 H), 2.82 (m, 2 H), 3.03−3.12 (m, 1 H), 4.08−4.14 (m, 1 H), 4.73 (m, 1 H), 6.45 (t, 1 H, J = 6 Hz), 7.48−7.62 (m, 3 H), 7.98−8.04 (m, 2 H), 8.20 (s, 1 H), 8.80 (s, 1 H) and 8.92 (s, br, 1 H); ^{13}C NMR ($CDCl_3$) δ 27.3, 40.1, 73.4, 85.0, 87.9, 123.9, 128.3, 129.2, 133.3, 133.9, 149.8, 151.8, 152.9 and 165.5; mass spectrum (FABMS) *m/z* 372.113 (M + H)$^+$ ($C_{17}H_{18}N_5O_3S$ requires 372.113).

***N*^{6}-Benzoyl-2′,5′-dideoxy-5′-[(4,4′-dimethoxytriphenylmethyl)thio]-adenosine (3a).** To a solution containing 300 mg (0.80 mmol) of **20** in 5 mL of pyridine was added 272 mg (0.80 mmol) of dimethoxytrityl chloride in 2 mL of pyridine at 0 °C. The combined solution was stirred for 1 h and was then concentrated under diminished pressure to afford a residue which was purified by flash chromatography on a silica gel column (20 cm × 3 cm). Elution with 95:5 CH_2Cl_2−methanol afforded **3a** as a colorless foam: yield 490 mg (91%); mp 120−125 °C; silica gel TLC R_f 0.50 (95:5 CH_2Cl_2−methanol); ^{1}H NMR ($CDCl_3$) δ 1.77 (br s, 1 H), 2.38−2.50 (m, 1 H), 2.58 (dd, 1 H, J = 6, 12 Hz), 2.69 (dd, 1 H, J = 6, 12 Hz), 2.78−2.86 (m, 1 H), 3.74 (s, 6 H), 3.86 (m, 1 H), 4.33−4.40 (m, 1 H), 6.43 (t, 1 H, J = 7 Hz), 6.78 (d, 4 H, J = 10.5 Hz), 7.18−7.40 (m, 9 H), 7.48−7.63 (m, 3 H), 8.00 (d, 2 H, J = 9 Hz), 8.09 (s, 1 H), 8.72 (s, 1 H) and 8.85 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 35.1, 39.7, 55.6, 66.6, 74.1, 85.2, 86.2, 113.6, 123.9, 127.2, 128.3, 129.2, 129.7, 131.0, 133.2, 134.0, 137.1, 142.2, 145.4, 152.9, 158.6 and 165.1; mass spectrum (FABMS) *m/z* 674.244 (M + H)$^+$ ($C_{38}H_{36}N_5O_5S$ requires 674.244).

***N*^{6}-Benzoyl-2′,5′-dideoxy-5′-[(4,4′-dimethoxytriphenylmethyl)thio]-adenosine 3′-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (3b).** To a stirred solution containing 60 mg (0.089 mmol) of **3a** in 1 mL of CH_2Cl_2 was added successively at room temperature 45 μL (0.23 mmol) of *N*,*N*-diisopropylethylamine and 19.8 μL (0.089 mmol) of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite. The reaction mixture was stirred at room temperature for 1 h and then diluted with 100 mL of ethyl acetate. The solution was washed with 10 mL of saturated $NaHCO_3$ solution and dried over $MgSO_4$. The dried solution was concentrated under diminished pressure to afford a residue which was purified by flash chromatography on a silica gel column (15 cm × 2 cm). Elution with 45:45:10 CH_2Cl_2−ethyl acetate−Et_3N afforded monomer **3b** as a colorless foam: yield 68 mg (86%) of a 1:1 mixture of diastereomers; silica gel TLC R_f 0.70 (45:45:10 CH_2Cl_2−ethyl acetate−Et_3N); ^{1}H NMR ($CDCl_3$) δ 1.14−1.19 (m, 12 H), 2.48−2.67 (m, 5 H), 2.82−2.91 (m, 1 H), 3.52−3.74 (m, 4 H), 3.77 (s, 3 H), 3.78 (s, 3 H), 4.10−4.18 (m, 1 H), 4.49−4.56 (m, 1 H), 6.31−6.40 (m, 1 H), 6.78 (d, 2 H, J = 4 Hz), 6.79 (d, 2 H, J = 4 Hz), 7.16−7.19 (m, 9 H), 7.48−7.63 (m, 3 H), 8.02 (d, 2 H, J = 7 Hz), 8.16 (s, 1 H), 8.72 (s, 1 H) and 8.87 (s, 1 H); mass spectrum (FABMS) *m/z* 874.351 (M + H)$^+$ ($C_{47}H_{53}N_7O_6PS$ requires 874.352).

***N*^{6}-Benzoyl-3′-*O*-(*tert*-butyldiphenylsilyl)-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-2′-deoxyadenosine (22).** To a solution containing 1.0 g (1.52 mmol) of nucleoside **21** and 155 mg (2.28 mmol) of imidazole in 15 mL of CH_2Cl_2 was added 433 μL (1.66 mmol) of *tert*-butyldiphenylsilyl chloride. The solution was stirred at room temperature for 12 h and then diluted with 250 mL of ethyl acetate. The reaction mixture was washed successively with 50 mL of saturated $NaHCO_3$ and 50 mL of brine, then dried ($MgSO_4$). The dried solution was concentrated under diminished pressure to afford a residue which was purified by flash chromatography on a silica gel column (20 cm × 3 cm). Elution with 3:1 ethyl acetate−hexanes afforded **22** as a white powder: yield 1.09 g (78%); mp 102−105 °C; silica gel TLC R_f 0.60 (3:1 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 1.13 (s, 9 H), 2.41−2.50 (m, 1 H), 2.52−2.65 (m, 1 H), 3.00 (dd, 1 H, J = 4.5, 10.5 Hz), 3.07 (dd, 1 H, J = 4.5, 10.5 Hz), 3.72 (s, 6 H), 4.22−4.28 (m, 1 H), 4.60−4.66 (m, 1 H), 6.52 (t, 1 H, J = 6 Hz), 6.68 (d, 4 H, J = 9 Hz), 7.08−7.74 (m, 22 H), 7.98 (d, 2 H, J = 10 Hz), 8.02 (s, 1 H), 8.70 (s, 1 H) and 8.82 (s, 1 H); mass spectrum (FABMS) *m/z* 896.381 (M + H)$^+$ ($C_{54}H_{54}N_5O_6Si$ requires 896.385).

***N*^{6}-Benzoyl-3′-*O*-(*tert*-butyldiphenylsilyl)-2′-deoxyadenosine (23).** A solution containing 1.06 g (1.18 mmol) of nucleoside **22** in 30 mL of 80% acetic acid was stirred at room temperature for 15 min. The solution was adjusted to pH 7 with saturated Na_2CO_3 solution, then diluted with 250 mL of ethyl acetate. The reaction mixture was washed successively with 50 mL of saturated $NaHCO_3$ solution and 50 mL of brine and was then dried over $MgSO_4$. The dried solution was concentrated under diminished pressure to afford a residue which was purified by flash chromatography on a silica gel column (20 cm × 3 cm). Elution with 3:1 ethyl acetate−hexanes afforded **23** as a colorless foam: yield 538 mg (77%); mp 98−100 °C; silica gel TLC R_f 0.30 (3:1 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 1.15 (s, 9 H), 2.31 (dd, 1 H, J = 5, 13 Hz), 2.89−2.99 (m, 1 H), 3.71−3.75 (m, 2 H), 4.16 (s, 1 H), 4.53 (d, 1 H, J = 5 Hz), 6.42 (dd, 1 H, J = 5.5, 11.5 Hz), 7.37−7.73 (m, 13 H), 7.99−8.02 (m, 2 H), 8.08 (s, 1 H), 8.72 (s, 1 H) and 8.93 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 19.5, 27.4, 41.5, 63.2, 75.2, 88.0, 90.4, 124.9, 128.3, 129.1, 130.5, 133.1, 133.5, 133.6, 133.9, 136.1, 142.9, 150.6, 151.2, 152.3 and 165.3; mass spectrum (FABMS) *m/z* 594.255 (M + H)$^+$ ($C_{33}H_{35}N_5O_4Si$ requires 594.254).

***N*^{6}-Benzoyl-3′-*O*-(*tert*-butyldiphenylsilyl)-2′-deoxy-5′-*O*-(*p*-toluenesulfonyl)adenosine (24).** To a solution containing 180 mg (0.30 mmol) of nucleoside **23** in 2 mL of pyridine was added 68.4 mg (0.36 mmol) of *p*-toluensulfonyl chloride in 0.50 mL of pyridine at 0 °C. The solution was stirred at room temperature for 3 h, then diluted with 250 mL of ethyl acetate. The reaction mixture was washed successively with 50 mL of saturated $NaHCO_3$ and 50 mL of brine, then dried over $MgSO_4$. The dried solution was concentrated under diminished pressure to afford a residue, which was purified by flash chromatography on a silica gel column (25 cm × 3 cm). Elution with 95:5 CH_2Cl_2−methanol afforded tosylate **24** as a colorless foam: yield 190 mg (85%); mp 83−85 °C; silica gel TLC R_f 0.50 (95:5 CH_2Cl_2−methanol); ^{1}H NMR ($CDCl_3$) δ 1.09 (s, 9 H), 2.37 (s, 3 H), 2.41−2.47 (m, 1 H), 2.56−2.58 (m, 1 H), 3.68 (dd, 1 H, J = 4, 12 Hz), 3.90 (dd, 1 H, J = 4, 12.5 Hz), 4.16 (s, 1 H), 4.52−4.60 (m, 1 H), 6.48 (dd, 1 H, J = 1.5, 6.5 Hz), 7.19 (d, 2 H, J = 9 Hz), 7.33−7.62 (m, 15 H), 7.72−7.86 (m, 2 H), 8.09 (s, 1 H) and 8.50 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 19.4, 22.0, 27.3, 40.5, 69.1, 74.1, 85.2, 85.5, 123.8, 128.2, 128.4, 128.5, 129.3, 130.2, 130.6, 130.7, 132.7, 133.1, 133.2, 134.0, 136.0, 136.1, 141.9, 145.4, 149.9, 152.9 and 164.9; mass spectrum (FABMS) *m/z* 748.265 (M + H)$^+$ ($C_{40}H_{41}N_5O_6SiS$ requires 748.262).

5′-Azido-*N*^{6}-benzoyl-3′-*O*-(*tert*-butyldiphenylsilyl)-2′,5′-dideoxyadenosine (25). A solution containing 170 mg (0.22 mmol) of tosylate **24** and 52 mg (0.80 mmol) of NaN_3 in 5 mL of DMF was stirred at 100 °C for 15 min. The solution was cooled to room temperature and diluted with 200 mL of ethyl acetate. The reaction mixture was washed with 50 mL of saturated $NaHCO_3$ solution and then dried over $MgSO_4$. The solution was concentrated under diminished pressure and the residue was coevaporated with portions of methanol. Precipitation of the product from ethyl acetate−hexanes afforded **25** as a white foam: yield 130 mg (95%); mp 112−115 °C; silica gel TLC R_f 0.70 (3:1 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 1.13 (s, 9 H), 2.55−2.63 (m, 1 H), 2.69−2.81 (m, 1 H), 3.10 (dd, 1 H, J = 3, 13 Hz), 3.33 (dd, 1 H, J = 5, 13 Hz), 4.18 (s, 1 H), 4.61 (s, 1 H), 6.55 (t, 1 H, J = 6 Hz), 7.40−7.78 (m, 13 H), 8.03−8.12 (m, 2 H) 8.22 (s, 1 H), 8.80 (s, 1 H) and 9.50 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 19.4, 27.3, 40.6, 52.2, 74.0, 85.0, 86.3, 124.1, 128.3, 128.4, 129.1, 130.6, 130.7, 133.0, 133.2, 133.3, 134.0, 136.1, 142.1, 150.1, 151.9, 152.9 and 165.3; mass spectrum FABMS *m/z* 619.257 (M + H)$^+$ ($C_{33}H_{35}N_8O_3Si$ requires 619.260).

5′-Amino-*N*^{6}-benzoyl-3′-*O*-(*tert*-butyldiphenylsilyl)-2′,5′-dideoxyadenosine (26). A solution containing 130 mg (0.21 mmol) of

nucleoside **25** and 45 mg of 5% palladium-on-carbon in 10 mL of ethanol was stirred under 1 atm H_2 at room temperature for 8 h. The catalyst was removed by filtration and the filtrate was concentrated under diminished pressure to afford a residue which was purified by flash chromatography on a silica gel column (15 cm × 3 cm). Elution with 9:1 CH_2Cl_2−methanol afforded **26** as a colorless powder: yield 100 mg (80%); mp 81−83 °C; silica gel TLC R_f 0.30 (9:1 CH_2Cl_2−methanol); ^{1}H NMR ($CDCl_3$) δ 1.13 (s, 9 H), 2.30 (dd, 1 H, J = 6, 11.5 Hz), 2.90−3.03 (m, 1 H), 3.22 (d, 1 H, J = 12 Hz), 3.75 (dd, 1 H, J = 12 Hz), 4.08 (s, 1 H), 4.76 (d, 1 H, J = 6 Hz), 5.60 (d, 2 H, J = 12 Hz), 6.44 (dd, 1 H, J = 6, 12 Hz), 7.35−7.75 (m, 13 H), 8.00 (d, 2 H, J = 9 Hz), 8.10 (s, 1 H), 8.72 (s, 1 H) and 8.93 (s, 1 H); ^{13}C NMR (CD_3OD) δ 18.9, 26.5, 38.7, 41.8, 75.2, 84.6, 86.2, 123.4, 128.2, 128.26, 128.4, 128.8, 130.4, 130.5, 133.0, 133.1, 133.9, 135.9, 144.0, 149.5, 151.5, 154.0 and 166.9; mass spectrum (FABMS) *m/z* 593.272 $(M + H)^+$ ($C_{33}H_{37}N_6O_3Si$ requires 593.270).

***N*^{6}-Benzoyl-3′-*O*-(*tert*-butyldiphenylsilyl)-2′,5′-dideoxy-5′-[(4-methoxytriphenylmethyl)amino]adenosine (27).** To a solution containing 70 mg (0.11 mmol) of nucleoside **26** in 2 mL of pyridine was added slowly 43.6 mg (0.14 mmol) of methoxytrityl chloride in 0.5 mL of pyridine at 0 °C. The combined solution was stirred at room temperature for 1 h and then partitioned between 25 mL of saturated $NaHCO_3$ solution and 100 mL of ethyl acetate. The organic layer was separated, washed with 25 mL of brine and then dried over $MgSO_4$. The dried solution was concentrated under diminished pressure to afford a residue which was purified by flash chromatography on a silica gel column (20 cm × 3 cm). Elution with 95:5 CH_2Cl_2−methanol afforded **27** as a colorless powder: yield 87 mg (91%); mp 112−115 °C; silica gel TLC R_f 0.70 (95:5 CH_2Cl_2−methanol); ^{1}H NMR ($CDCl_3$) δ 1.11 (s, 9 H), 2.12−2.25 (m, 2 H), 2.40 (ddd, 1 H, J = 1.5, 5.5, 13 Hz), 2.65 (s, 1 H), 2.91−3.00 (m, 1 H), 3.75 (s, 3 H), 4.25−4.30 (m, 1 H), 4.74−4.78 (m, 1 H), 6.39 (dd, 1 H, J = 5.5, 8 Hz), 6.69 (d, 2 H, J = 9 Hz), 7.13−7.68 (m, 25 H), 7.88 (s, 1 H), 7.99 (d, 2 H, J = 7 Hz), 8.17 (s, 1 H) and 8.84 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 19.5, 27.4, 40.7, 46.1, 55.6, 70.6, 75.2, 85.9, 88.7, 113.5, 124.3, 126.6, 128.2, 128.3, 128.9, 129.0, 129.2, 130.2, 130.5, 133.1, 133.5, 133.7, 134.1, 136.1, 138.2, 142.5, 146.4, 146.5, 150.0, 151.8, 152.9, 158.2 and 165.0; mass spectrum (FABMS) *m/z* 865.387 $(M + H)^+$ ($C_{53}H_{53}N_6O_4Si$ requires 865.389).

***N*^{6}-Benzoyl-2′,5′-dideoxy-5′-[(4-methoxytriphenylmethyl)amino]-adenosine (4a).** To a solution containing 83 mg (0.096 mmol) of nucleoside **27** in 1 mL of THF was added 105 μL (0.10 mmol) of TBAF as a 1 M solution in THF. The reaction mixture was stirred for 5 h at room temperature, then the solution was concentrated under diminished pressure to afford a residue which was purified by flash chromatography on a silica gel column (15 cm × 3 cm). Elution with 9:1 CH_2Cl_2−methanol afforded nucleoside analogue **4a** as a colorless foam: yield 48 mg (81%); mp 105−107 °C; silica gel TLC R_f 0.70 (9:1 CH_2Cl_2−methanol); ^{1}H NMR ($CDCl_3$) δ 2.41 (ddd, 1 H, J = 3.5, 6, 9 Hz), 2.48 (d, 2 H, J = 4 Hz), 2.75 (s, 1 H), 3.05 (2 t, 1 H, J = 6.5 Hz), 3.54 (s, 1 H), 3.73 (s, 3 H), 4.16 (d, 1 H, J = 3 Hz), 4.80−4.90 (m, 1 H), 6.31 (t, 1 H, J = 6 Hz), 6.75 (d, 2 H, J = 9 Hz), 7.11−7.59 (m, 15 H), 7.91 (s, 1 H), 7.98 (d, 2 H, J = 7.5 Hz), 8.28 (s, 1 H) and 9.23 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 40.5, 46.2, 55.6, 70.7, 73.4, 85.7, 87.9, 113.6, 124.0, 126.7, 128.2, 129.0, 129.2, 130.2, 133.2, 134.0, 138.2, 142.4, 146.3, 146.4, 149.9, 151.7, 153.0, 158.3 and 165.2.

***N*^{6}-Benzoyl-2′,5′-dideoxy-5′-[(4-methoxytriphenylmethyl)amino]-adenosine 3′-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (4b).** To a solution containing 43 mg (0.069 mmol) of nucleoside **4a** in 2 mL of CH_2Cl_2 was added successively at room temperature 32.8 μL (0.19 mmol) of *N*,*N*-diisopropylethylamine and 15.3 μL (0.069 mmol) of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite. The solution was stirred for 1 h and then diluted with 50 mL of ethyl acetate. The reaction mixture was washed with 15 mL of saturated $NaHCO_3$ and then dried over $MgSO_4$. The dried solution was concentrated under diminished pressure to afford a residue which was purified by flash chromatography on a silica gel column (15 cm × 3 cm). Elution with 45:45:10 CH_2Cl_2−ethyl acetate−Et_3N afforded monomer **4b** as a colorless foam: yield 52 mg (91%) as a 1:1 mixture of diastereomers; mp 96−98 °C; silica gel TLC R_f 0.50 (5:45:10 CH_2Cl_2−ethyl acetate−Et_3N); ^{1}H NMR ($CDCl_3$) δ 1.14−1.29 (m, 12 H), 2.42−2.70 (m, 5 H), 2.95 (m, 1 H), 3.30 (m, 1 H), 3.60−3.95 (m, 4 H), 3.763 (s, 1.5 H), 3.765 (s, 1.5 H), 4.38−4.47 (m, 1 H), 4.92−5.06 (m, 1 H), 6.31−6.36 (m, 1 H), 6.72−6.74 (m, 2 H), 7.14−7.63 (m, 15 H), 7.98−8.05 (m, 3 H), 8.15 (s, 0.5 H), 8.23 (s, 0.5 H) and 8.93 (s, 1 H); mass spectrum (FABMS) *m/z* 827.380 $(M + H)^+$ ($C_{46}H_{52}N_8O_5Si$ requires 827.380).

Enzyme Purification. Calf thymus DNA topoisomerase I was isolated and purified by slight modification of a published procedure.[44] The isolated protein exhibited two major bands ($M_r \approx$ 96 000 and 82 000) when analyzed by SDS-polyacrylamide gel electrophoresis and visualization of the protein by silver staining. The heterogeneity of the isolated topoisomerase I can be attributed to proteolysis during the isolation procedures.[45] The purified protein had a specific activity of 1.4×10^7 units/mg protein.

Oligonucleotide Substrates. Synthetic oligonucleotides were purchased from Cruachem Inc. or synthesized on a Biosearch 8600 series DNA synthesizer using standard phosphoramidite chemistry.[25] The synthesized oligonucleotides were deblocked and cleaved from the solid support by treatment with concentrated NH_4OH at 55 °C for 12 h. The 5′-CH_2OH and -NH_2 acceptor oligomers were detritylated and purified by Nensorb chromatography according to the manufacturer's protocol. Removal of the MTr group was effected by treatment with 2% trifluoroacetic acid and the DMTr group with 0.5% trifluoroacetic acid. All oligonucleotides were purified on a preparative 20% denaturing polyacrylamide gel, and the DNA recovered by crush and soak, then by precipitation. The DNA was 5′-^{32}P end labeled with T4 polynucleotide kinase + [γ-^{32}P]ATP.[46]

Hybridization of Substrates. Oligonucleotides were hybridized in a solution (100 μL total volume) containing 10 mM Tris-HCl, pH 7.5, 40 mM NaCl, 5 mM $MgCl_2$ and 5 mM $CaCl_2$. The solution was heated to 80 °C for 5 min and cooled slowly to room temperature under ambient conditions over a period of ∼3 h. Due to the low DNA strand concentrations, hybridization mixtures contained 0.13 pmol of the labeled strand and a 100-fold excess of the unlabeled strands to ensure complete hybridization of the labeled DNA.

Formation of the Topoisomerase I−DNA Covalent Complex. The labeled duplex (6.5 fmol, 100,000 dpm) was incubated with topoisomerase I (8.8 ng) in reaction mixture (20 μL total volume) containing 10 mM Tris-HCl, pH 7.5, 40 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.5 mM EDTA and 0.5 mM DTT for 60 min at 37 °C (13:1 enzyme-duplex DNA).

Purification of the Topoisomerase I−DNA Covalent Intermediate. The topoisomerase I cleavage reaction mixture was applied to an FPLC Mono Q HR 5/5 column equilibrated in 20 mM Tris-HCl, pH 7.5, containing 0.5 mM EDTA, followed by a 30-mL wash with the same buffer. Elution was effected with a 30-mL linear gradient of NaCl (0 → 1 M) in the same buffer at a flow rate of 1 mL/min. One-mL fractions were collected and an aliquot of each fraction (100 μL) was utilized for scintillation counting. Typically, 20 individual topoisomerase I cleavage reactions were combined and applied to the column.

Proteolysis and Strand Transfer of the Topoisomerase I−DNA Complex. The fractions containing 5′-^{32}P end labeled covalent complex (2.9×10^{-16} mol, 4400 dpm) were incubated with 1.25×10^{-11} mol of the indicated acceptor oligonucleotides promptly after separation by FPLC in a 110 μL (total volume) reaction mixture containing 20 mM Tris-HCl, pH 7.5, 400 mM NaCl, 0.5 mM EDTA, 5 mM $MgCl_2$ and 1 mM DTT. The thiol acceptor oligonucleotide was reduced immediately prior to use with 10 mM DTT. The reactions were incubated for 60 min at 37 °C followed by proteolysis with 1 mg/mL proteinase K (1 h; 37 °C) and lyophilization. The samples were reconstituted in 30 μL of 20 mM Tris-HCl, pH 7.5 and 15 μL of loading solution. The reactions were heat-denatured at 90 °C for 5 min and quick chilled on ice; 15 μL was applied to a 20% denaturing PAGE.

Effect of pH on Topoisomerase I-Mediated Ligation. The 5′-^{32}P end labeled covalent complex (2.0×10^{-16} mol, 3000 dpm) in 58 μL of 20 mM Tris-HCl, pH 7.5, containing 400 mM NaCl and 0.5 mM EDTA was diluted with an equal volume of 100 mM Tris-HCl at pH 7.5, 8.0, 8.5 or 9.0. $MgCl_2$ and DTT were added to a final concentrations of 5 mM, and 1 mM, respectively, and the acceptor oligonucleotide (6.25×10^{-11} mol) was added to initiate the reaction. The reactions were incubated for 60 min at 37 °C followed by proteolysis with 1 mg/mL proteinase K (1 h; 37 °C) and lyophilization. The samples were reconstituted in 30 μL of 20 mM Tris-HCl, pH 7.5

and 15 μL of loading solution. The reactions were heat-denatured at 90 °C for 5 min and quick chilled on ice; 15 μL was applied to a 20% denaturing PAGE.

Time Course of Topoisomerase I-Mediated Ligation. The 5′-^{32}P end labeled covalent complex (3.25×10^{-16} mol, 5000 dpm) was incubated with 6.25×10^{-12} mol of the indicated acceptor oligonucleotide in a reaction mixture (70 μL total volume) containing 20 mM Tris-HCl, pH 7.5, 400 mM NaCl, 0.5 mM EDTA, 5 mM $MgCl_2$ and 1 mM DTT. The reactions were incubated for 5, 30 or 60 min at 37 °C, quenched by the addition of 1% SDS and proteolyzed with 1 mg/mL proteinase K (1 h; 37 °C). The reactions were lyophilized and then dissolved in 30 μL of 20 mM Tris-HCl, pH 7.5 and 15 μL of loading solution. The reactions were heat denatured at 90 °C for 5 min and quick chilled on ice; 15 μL of each was applied to a 20% denaturing PAGE.

Exonuclease III Digestion. Reaction mixtures consisted of 10 μL (total volume) of 50 mM Tris-HCl, pH 8.0, containing 5 mM $MgCl_2$, 1 mM DTT, 6000 dpm of 5′-^{32}P end labeled duplex DNA and 2 μg of calf thymus DNA. The reactions were initiated by the addition of 64 units of exonuclease III. After 20 min, the reactions were terminated by the addition of 2 μL of 200 mM EDTA and 6 μL of loading solution. The reactions were heat denatured at 90 °C for 5 min and quick chilled on ice; 5 μL of each was applied to a 20% denaturing PAGE.

DNA Strand Scission by Iodine. To a 10 μL aqueous solution of 5′-^{32}P end labeled DNA (5000 dpm) was added 10 μL of 100 mM iodine in pyridine. After 2 h at room temperature the reactions were dried under diminished pressure, then redissolved in 5 μL of water and 3 μL of loading solution. The reactions were heat denatured at 90 °C for 5 min and quick chilled on ice; 5 μL of each was applied to a 20% denaturing PAGE.

DNA Strand Scission by Acetic Acid. The 5′-^{32}P end labeled DNA (1500 dpm) was treated with 15% acetic acid for 15 h at room temperature. The reactions were dried under diminished pressure, then redissolved in 5 μL of water and 3 μL of loading solution. The reactions were heat denatured at 90 °C for 5 min and quick chilled on ice; 5 μL of each was applied to a 20% denaturing PAGE.

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Supporting Information Available: Data illustrating topoisomerase I-mediated ligation of **I** and **II**, DNA sequencing analysis of topoisomerase I-mediated ligation of **I**, the effect of Mg^{2+} and Mn^{2+} on the ligation of **I** and **III**, ligation of the modified acceptors as a function of pH, and the results of exonuclease III digestion of products formed in the presence of oligonucleotides **I** and **II** (9 pages). See any current masthead page for ordering and Internet access instructions.

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