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Inhibition of Human Topoisomerases I and II by Simocyclinone D8

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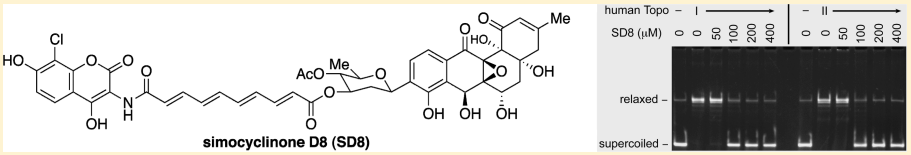
Supporting Information



ABSTRACT: Simocyclinone D8 is an antibiotic isolated from *Streptomyces antibioticus* Tü 6040 that inhibits the supercoiling activity of DNA gyrase. It also exhibits an inhibitory effect on human topoisomerase II and an antiproliferative activity against some cancer cell lines. Our biochemical studies have revealed that simocyclinone D8 can inhibit the catalytic activity of human topoisomerase I. Thus, simocyclinone D8 is a dual catalytic inhibitor of human topoisomerases I and II.

DNA topoisomerases are responsible for resolving topological problems that arise from the double-helical nature of DNA during various cellular processes such as DNA replication, transcription, and chromosome segregation.[1−4] Topoisomerases alter the topological state of the DNA through a cycle of DNA cleavage, strand transfer, and religation. There are five types of topoisomerases: IA, IB, IC, IIA, and IIB.[1−4] Human topoisomerase I (Topo I) is a type IB enzyme that cleaves only one strand of the DNA duplex, whereas human topoisomerase II (Topo II) is a type IIA enzyme that cleaves both strands. Type IB topoisomerases are thought to have evolved from a common ancestor as tyrosine recombinases and are structurally distinct from other types of topoisomerases. Type IIA topoisomerases, from bacterial DNA gyrase and topoisomerase IV to human Topo II, are highly conserved.

Topoisomerases are the cellular targets of clinically important anticancer and antibacterial drugs, such as etoposide, doxorubicin, camptothecin (CPT), and fluoroquinolones.[5−9] These drugs shift the DNA cleavage/religation equilibrium and increase the steady-state level of topoisomerase−DNA covalent complexes. Formation of drug-induced topoisomerase−DNA covalent complexes leads to the generation of double-strand breaks and subsequent cell death. Because of their unique mode of action, these drugs are often referred to as topoisomerase poisons. Other topoisomerase-targeting drugs affect a different step during the catalytic cycle of topoisomerases and inhibit the catalytic activity of topoisomerases without poisoning them.[7−10] These drugs are referred to as topoisomerase catalytic inhibitors. For example, aminocoumarins are ATPase inhibitors, and aclarubicin blocks the binding of Topo II to DNA.

Simocyclinone D8 (SD8), an antibiotic isolated from *Streptomyces antibioticus* Tü 6040,[11−14] is also a topoisomerase catalytic inhibitor.[15,16] The initial study of SD8 has shown that SD8 is active against Gram-positive bacteria, such as *Streptomyces viridochromogenes*, *Streptomyces coelicolor*, and *Bacillus brevis* [minimum inhibitory concentration (MIC) = 1−10 μg/mL], but not against Gram-negative bacteria, such as *Escherichia coli*.[11] A more recent study has demonstrated, however, that SD8 is active against certain clinical isolates of *E. coli* (MIC = 0.78−3.12 μg/mL), although the MIC values for SD8 are 3−12-fold higher than those for ciprofloxacin.[12] Biochemical studies of SD8 have shown that the 50% inhibitory concentration (IC_{50}) for SD8 in the supercoiling assay with *E. coli* DNA gyrase is 6−7-fold lower than that for ciprofloxacin[15,16] and that SD8 inhibits the supercoiling activity of *E. coli* DNA gyrase much more effectively (the IC_{50} value is approximately 650-fold lower) than the decatenation activity of *E. coli* topoisomerase IV.[16] SD8 contains a chlorinated aminocoumarin and an angucyclic polyketide, linked by a tetraene and D-olivose.[11,13,14,17] Binding of both aminocoumarin and polyketide moieties to the DNA binding domain of DNA gyrase is essential for its strong, selective inhibition.[18] In a previous study,[19] we demonstrated that SD8 can inhibit the catalytic activity of human Topo II and concluded that the antiproliferative activity of SD8 against certain cancer cell lines is due to its inhibitory effect on Topo II activity.

Because SD8 has exhibited modest antiproliferative activities against several cancer cell lines,[11,19] we continued our studies of the effects of SD8 on human topoisomerases. We found that SD8 inhibited the relaxation activity of human Topo I (Figure 1). This observation was unexpected because SD8 binds to the conserved DNA binding domain of DNA gyrase,[15,18] and unlike human Topo II, human Topo I does not have any structural similarities to DNA gyrase.[1−4] To confirm this unexpected observation, we repeated the relaxation assay using calf thymus Topo I, as well as three independent preparations of SD8, and

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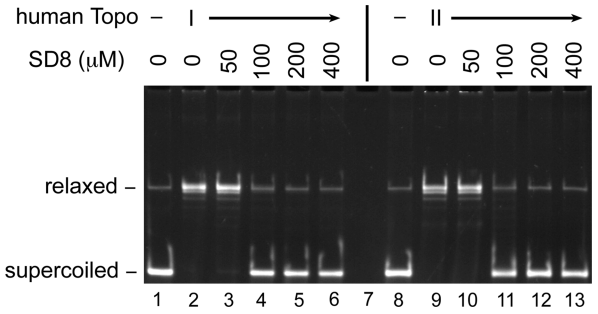

Figure 1. SD8 can inhibit the relaxation activity of either human Topo I or human Topo II. The inhibitory effects of SD8 on the relaxation activity of either human Topo I or human Topo II were measured.

found that SD8 could inhibit the relaxation activity of Topo I (Figure 1; data not shown). Each assay described in this study was repeated at least twice with human Topo I and once with calf thymus Topo I, and essentially identical results were obtained. Representative results with human Topo I are shown in the figures.

The inhibitory effect of SD8 on Topo I was not significantly affected by either magnesium (up to 1 mM) or salt (up to 100 mM NaCl or 200 mM potassium glutamate) (data not shown). The IC_{50} values (60 ± 15 μM) varied slightly between experiments, and this appeared to be due to variation in the specific activity among preparations of the topoisomerases, rather than to variation in the activity of SD8 preparations themselves. We found that the level of inhibitory effect of SD8 on human Topo I was essentially identical to that on human Topo II in the relaxation assay (Figure 1). The IC_{50} value of SD8 for Topo I was approximately 2-fold higher than that of CPT for Topo I, whereas the IC_{50} value of SD8 for human Topo II was approximately 2-fold lower than that of etoposide for human Topo II.[19] These results showed that SD8 could act as a dual catalytic inhibitor of human Topo I and Topo II. It is likely that inhibition of the catalytic activities of both topoisomerases contributes to the antiproliferative activity of SD8.

To determine if SD8 could poison Topo I, we conducted a DNA cleavage assay and found that SD8 did not stimulate the DNA cleavage activity of Topo I (Figure 2). Furthermore, the presence of SD8 appeared to inhibit the DNA cleavage activity of human Topo I (Figure 2, compare lanes 4–6 with lane 2). These results demonstrated that SD8 inhibited the catalytic

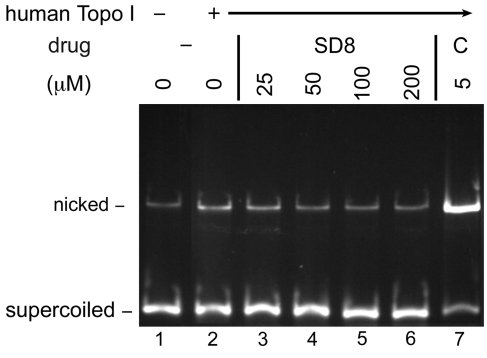

Figure 2. SD8 does not poison human Topo I. The DNA cleavage assay was performed to assess the effect of SD8 on the DNA cleavage activity of human Topo I. Lane 7 is a control reaction with 5 μM CPT (C).

activity of human Topo I without poisoning it, as was the case with human Topo II.[19]

SD8 binds to DNA gyrase and prevents DNA gyrase from binding to DNA; therefore, it inhibits the stimulation of the DNA cleavage activity of DNA gyrase by a fluoroquinolone.[15] The ability of SD8 to inhibit the effect of a topoisomerase poison on a topoisomerase can be directly assessed by a two-step DNA cleavage assay.[15,16] If SD8 can block the binding of either human Topo I or human Topo II to DNA, incubation of a human topoisomerase with SD8 prior to the addition of a topoisomerase poison would inhibit drug-induced, topoisomerase-catalyzed DNA cleavage. We performed the two-step DNA cleavage assay and found that this was the case (Figure 3A and B). Incubation of SD8 with either human Topo I (Figure 3A, lanes 4–6) or human Topo II (Figure 3B, lanes 4–6) and DNA prior to the addition of either CPT (to the Topo I reaction) or etoposide (to the Topo II reaction) completely inhibited DNA cleavage activity of the topoisomerase, suggesting that SD8 could prevent either Topo I or Topo II from binding to DNA. In contrast, when either Topo I and CPT (Figure 3A, lanes 1–3) or Topo II and etoposide (Figure 3B, lanes 1–3) were incubated with the DNA prior to the addition of SD8, we detected stimulation of the DNA cleavage activities of the topoisomerases by these topoisomerase poisons.

After the formation of human Topo I–CPT–DNA ternary complexes, incubation with SD8 lowered the amount of nicked DNA, which corresponds to the number of human Topo I–CPT–DNA ternary complexes, by approximately 40% when compared to incubation with either CPT or DMSO (Figure 3A, compare lane 1 with lanes 2 and 3). This contrasted with human Topo II, where incubation with SD8 did not affect the amount of linear DNA, which corresponds to the number of human Topo II–etoposide–DNA ternary complexes (Figure 3B, compare lane 1 with lanes 2 and 3). The distinct effects of SD8 on ternary complexes formed with human Topo I and Topo II became more evident when the second stage of the reaction was followed over a longer period of time (Figure 3C and D). The amount of nicked DNA decreased with time, and only about one-half (53 ± 7%) of ternary complexes remained after a 20 min incubation with SD8 (Figure 3C), whereas the amount of linear DNA did not decrease (107 ± 1% at 20 min) during the second stage of the reaction (Figure 3D). These results showed that SD8 could promote the disassociation of Topo I–CPT–DNA ternary complexes but not that of Topo II–etoposide–DNA ternary complexes.

It is possible, although unlikely, that human Topo I–CPT–DNA ternary complexes spontaneously disassociate and SD8 sequesters human Topo I during the second stage of the reaction. Alternatively, SD8 may interact with human Topo I in ternary complexes and actively promote their disassociation. To distinguish these possibilities, we performed a dilution experiment (Figure 4). Dilution of the reaction mixtures lowers the concentrations of Topo I, CPT, and the DNA to prevent the rebinding of Topo I to DNA once ternary complexes disassociate. Lowering of the enzyme and the drug concentrations may also cause the disassociation of Topo I–CPT–DNA ternary complexes. A 10-fold dilution with the dilution buffer containing no drug (reaction 2), CPT (reaction 3), SD8 (reaction 4), or both SD8 and CPT (reaction 5) decreased the amount of human Topo I–CPT–DNA ternary complexes by approximately 35%, 6%, 55%, and 46%, respectively (Figure 4B). Thus, approximately one-third of the ternary complexes disassociated as a result of the dilution

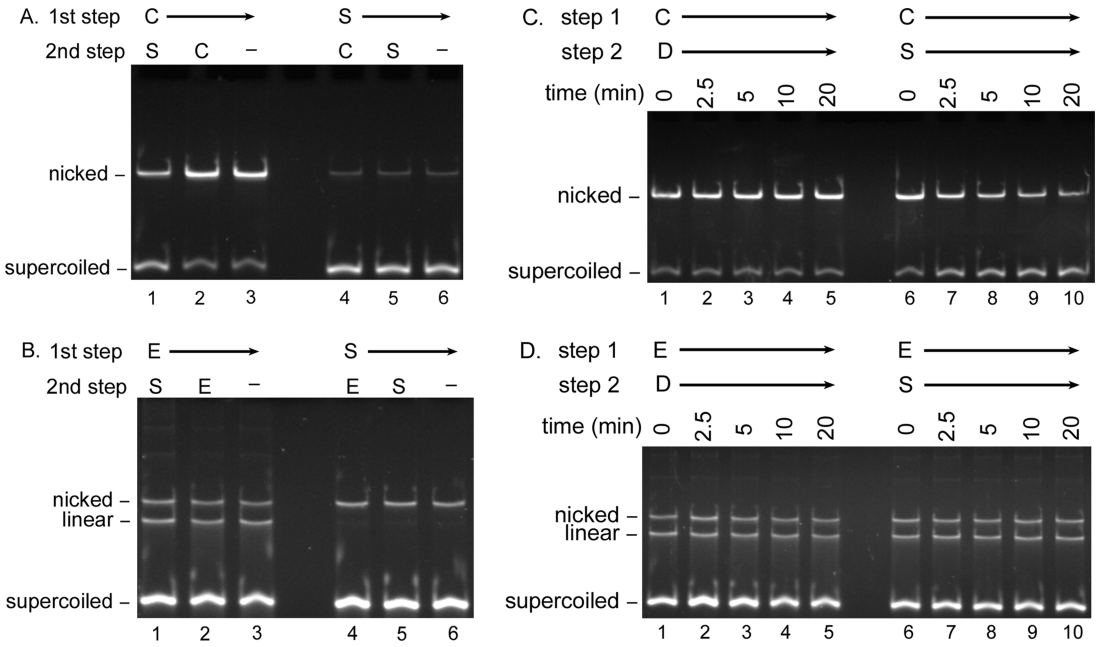

Figure 3. SD8 affects the stability of human Topo I–CPT–DNA ternary complexes but not that of human Topo II–etoposide–DNA ternary complexes. The ability of SD8 (S) to inhibit the activity of either CPT (C) against human Topo I (panel A) or etoposide (E) against human Topo II (panel B) was assessed using the two-step DNA cleavage assay. Ternary complexes were formed in the presence of either 7.5 μM CPT or 7.5 μM etoposide. Lanes 3 and 6 in both panels show DNA products after the first stage of the reaction (thus, the second step is indicated as "–"). Effect of SD8 (S) on the stability of either human Topo I–CPT (C)–DNA (panel C) or human Topo II–etoposide (E)–DNA (panel D) ternary complexes was examined by the modified two-step DNA cleavage assay. In the second stage of the reaction, either DMSO (D) or 150 μM SD8 (S) was added and aliquots were taken at the indicated times.

(Figure 4, compare reaction 2 with reaction 1). Maintaining the CPT concentration after dilution reduced the level of ternary complex disassociation (Figure 4, compare reaction 3 with reaction 2). The presence of SD8 after dilution significantly increased (by 20–40%) the level of ternary complex disassociation (Figure 4, compare reaction 4 with reaction 2, and reaction 5 with reaction 3), suggesting that SD8 can actively promote disassociation of the ternary complexes. Under the normal reaction conditions (i.e., no dilution), it is likely that Topo I–CPT–DNA ternary complexes are stable enough not to disassociate in significant numbers. Thus, the observed disassociation of human Topo I–CPT–DNA ternary complexes (Figure 3A and C) is likely promoted by the action of SD8 on Topo I in the ternary complexes.

Because of the structural similarities among type IIA topoisomerases,[1−4] it seems reasonable to assume that SD8 binds to DNA gyrase, topoisomerase IV, and human Topo II in a similar manner.[16,18] We compared amino acid sequences (BLAST, NCBI) between the aminocoumarin and polyketide binding pocket on the *E. coli* GyrA protein[18] and corresponding regions on either the *E. coli* ParC protein or the α form of human Topo II (data not shown). As reported previously,[18] all five amino acid residues in the polyketide binding pocket and five of the seven amino acid residues in the aminocoumarin binding pocket are identical between GyrA and ParC. There are two nonconserved changes between the aminocoumarin binding pockets of GyrA and ParC: the histidine residue at the 45 position and the arginine residue at the 91 position of GyrA are replaced by a glutamine and a leucine residue in ParC, respectively. SD8-resistant mutations are located at these positions in GyrA.[18] Four of the five amino acid residues in the polyketide binding pocket are identical between GyrA and human Topo II, and one amino acid difference is a conserved change (the arginine residue at the 47 position of GyrA is replaced by a lysine in human Topo II). In contrast, only two of the seven amino acid residues (the lysine residue at the 42 position and the glycine residue at the 170 position in GyrA) in the aminocoumarin binding pocket are identical between GyrA and human Topo II, and the five amino acid differences are nonconserved changes. The amino acid residue that corresponds to the histidine residue at the 45 position in GyrA is a glutamine in human Topo II, and the H45Q mutation in GyrA confers SD8 resistance to DNA gyrase.[18] Thus, the differences in amino acid residues in the aminocoumarin binding pocket among these type IIA topoisomerases are likely to be the key factors that determine the SD8 sensitivities of these enzymes.

Human Topo I does not have any structural similarities with type IIA topoisomerases.[1−4] Therefore, at this point, it is not clear how SD8 binds to human Topo I, and further studies are necessary to identify the SD8 binding site on human Topo I. Our current study demonstrates that SD8 acts as a dual catalytic inhibitor of human Topo I and Topo II and that SD8 may serve as a useful lead compound for the development of novel anticancer drugs.

■ EXPERIMENTAL SECTION

General Experimental Procedures. SD8 was isolated and purified from *S. antibioticus* Tü 6040[16] using the previously described procedures (Supporting Information).[11] The final preparations of SD8 were analyzed by analytical reversed-phase high-performance liquid chromatography and nuclear magnetic resonance to confirm that only SD8 is present.[16] A preparation of SD8 kindly provided by Dr. Hans-Peter Fiedler (Universität Tübingen) was also used. CPT and etoposide were purchased from Topogen (Port Orange, FL, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Stock solutions (20 mM) of these compounds were prepared in 100% DMSO (solvent), and compounds were diluted in either 40% or 50% DMSO.

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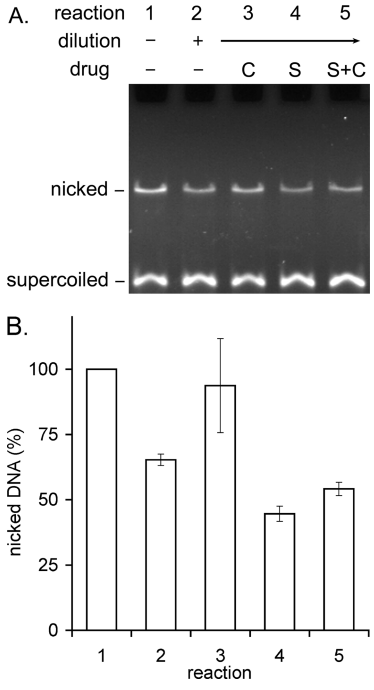

Figure 4. SD8 promotes disassociation of human Topo I–CPT–DNA ternary complexes. The dilution experiment was performed to examine the ability of SD8 to promote the disassociation of human Topo I–CPT–DNA ternary complexes. After the first step incubation with Topo I and 2.5 μM CPT (C), reaction mixtures were diluted 10-fold with dilution buffer containing no drug (–; reaction 2), 2.5 μM CPT (C; reaction 3), 160 μM SD8 (S; reaction 4), or both 2.5 μM CPT and 160 μM SD8 (S+C; reaction 5). Representative results are shown in panel A, and the average value and the absolute deviation from two independent assays using human Topo I are shown in panel B. The first lane (reaction 1) in panel A is a control showing the DNA products prior to the dilution, and the amount of nicked DNA after dilution (reactions 2–5) was normalized to the amount of nicked DNA prior to the dilution (reaction 1) in panel B.

The final concentration of DMSO in the reaction mixtures was either 2% or 2.5%; DMSO alone at these concentrations did not affect the activities of topoisomerases used in this study.

Both human Topo I and Topo II were purchased from Topogen. Calf thymus Topo I was from Life Technologies (Grand Island, NY, USA). Negatively supercoiled pBR322 DNA was purchased from New England Biolabs (Ipswich, MA, USA), and relaxed plasmid DNA was prepared by incubating the negatively supercoiled DNA with calf thymus Topo I.

Relaxation Assay for Topo I. Relaxation reaction mixtures (20 μL) for Topo I contained 50 mM Tris-HCl (pH 7.5 at 23 °C), 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 50 μg/mL bovine serum albumin (BSA), 0.3 μg of negatively supercoiled pBR322 DNA, either 1 or 2 units of human Topo I (or 0.5 unit of calf thymus Topo I), and the indicated concentrations of SD8. Reaction mixtures were incubated at 37 °C for either 30 min (with 1 unit of human Topo I) or 15 min (with either 2 units of human Topo I or 0.5 unit of calf thymus Topo I) and terminated by adding EDTA to a final concentration of 25 mM and incubating at 37 °C for 5 min. The DNA products were analyzed by electrophoresis through vertical 1.2% SeaKem ME agarose (Lonza, Rockland, ME, USA) gels (14 × 10 × 0.3 cm) at 2 V/cm for 15 h in a running buffer of 50 mM Tris-HCl (pH 7.9 at 23 °C), 40 mM sodium acetate, and 1 mM EDTA (TAE buffer). Gels were stained with ethidium bromide, then photographed, and quantified using a Stratagene Eagle Eye gel documentation system (Agilent Technologies, Santa Clara, CA, USA).

Relaxation Assay for Human Topo II. Relaxation reaction mixtures (20 μL) contained 50 mM Tris-HCl (pH 8.0 at 23 °C), 10 mM $MgCl_2$, 200 mM potassium glutamate, 10 mM DTT, 50 μg/mL BSA, 1 mM ATP, 0.3 μg of negatively supercoiled pBR322 DNA, 2 units of human Topo II, and the indicated concentrations of SD8. Reaction mixtures were incubated at 37 °C for 30 min and terminated by adding EDTA to a final concentration of 25 mM and further incubating at 37 °C for 5 min. The DNA products were analyzed, and the gels were photographed and quantified as described in the previous paragraph.

DNA Cleavage Assays. The standard DNA cleavage reaction mixtures (20 μL) for Topo I contained 50 mM Tris-HCl (pH 7.5 at 23 °C), 1 mM $MgCl_2$, 1 mM DTT, 50 μg/mL BSA, 0.3 μg of relaxed plasmid DNA, 5 units of human Topo I (or 2 units of calf thymus Topo I), and the indicated concentrations of SD8 or CPT. Reaction mixtures were incubated at 37 °C for 10 min before stopping the reaction by addition of SDS to 1% and incubating at 37 °C for 5 min. Then, EDTA and proteinase K were added to a final concentration of 25 mM and 100 μg/mL, respectively, and the incubation was continued at 37 °C for 15 min. The DNA products were purified by extraction with phenol–chloroform–isoamyl alcohol (25:24:1, v/v/v) and then analyzed by electrophoresis through vertical 1.2% agarose gels at 2 V/cm for 12 h in a TAE buffer containing 0.5 μg/mL ethidium bromide. Gels were destained in water before being photographed and quantified using the Eagle Eye system.

The standard DNA cleavage reaction mixture (20 μL) for human Topo II contained 50 mM Tris-HCl (pH 8 at 23 °C), 10 mM $MgCl_2$, 10 mM DTT, 50 μg/mL BSA, 1 mM ATP, 0.3 μg of relaxed plasmid DNA, 5 units of human Topo II, and the indicated concentrations of SD8 or etoposide. Reaction mixtures were incubated at 37 °C for 10 min. SDS was added to a concentration of 1%, and the reaction mixtures were further incubated at 37 °C for 5 min to stop the DNA cleavage reaction. EDTA and proteinase K were then added to a final concentration of 25 mM and 100 μg/mL, respectively, and the incubation was continued for an additional 60 min at 50 °C. The DNA products were purified and analyzed as described in the previous section.

Two-Step DNA Cleavage Assay. The DNA cleavage reaction was increased in size by 4-fold (80 μL), and either Topo I and 150 μM SD8 or 7.5 μM CPT, or human Topo II and 150 μM SD8 or 7.5 μM etoposide were incubated at 37 °C for 5 min. Three aliquots (20 μL each) were taken after the first stage of the reaction. Either 7.5 μM CPT (for the reactions with Topo I) or 7.5 μM etoposide (for the reactions with Topo II) was added to one aliquot, 150 μM SD8 was added to another, and the reaction mixtures were incubated at 37 °C for 10 min. SDS was added to a concentration of 1% to stop the second stage of the reaction, and the incubation was continued at 37 °C for 5 min. EDTA and proteinase K were then added to a final concentration of 25 mM and 100 μg/mL, respectively, and the reaction mixtures were further incubated either at 37 °C for 15 min (for the reactions with Topo I) or at 50 °C for 60 min (for the reactions with Topo II). DMSO (to a final concentration of 2%) and SDS (to a final concentration of 1%) were added to a third aliquot immediately after the first stage of the reaction, and the reaction mixtures were processed in the same manner as the two other aliquots. The DNA products were purified and analyzed as described in the DNA cleavage assay for the Topo I section.

Two-step DNA cleavage assays described above were modified to assess the effects of SD8 on the ternary complexes formed with either Topo I or human Topo II. The reaction mixtures were as described above for two-step assays for both Topo I and Topo II except that the DNA cleavage reaction was increased in size by 6-fold (120 μL). For the first stage of the reaction, a pair of reaction mixtures containing either 7.5 μM CPT and Topo I or 7.5 μM etoposide and Topo II were incubated at 37 °C for 5 min. Then, 150 μM SD8 was added to one reaction mixture and DMSO (to a final concentration of 2%) was added to the other, and the second stage of the reaction was continued at 37 °C. At the indicated times, 20 μL aliquots were taken and mixed with SDS (to a final concentration of 1%). After a 5 min incubation, EDTA and proteinase K were added to a final concentration of 25 mM and 100 μg/mL, respectively, and the reaction mixtures were processed as described in the previous paragraph.

Dilution Experiment. The standard DNA cleavage reaction (20 μL) for Topo I was assembled as described in the DNA cleavage assay for the Topo I section, except that 0.6 μg of relaxed plasmid DNA, 2.5 μM CPT, and 5 units of human Topo I (or 2 units of calf thymus Topo I) were included. After a 5 min incubation at 37 °C, the reaction mixtures were diluted 10-fold with the dilution buffer, which is identical to the reaction buffer for Topo I DNA cleavage reactions except DNA and Topo I were omitted, containing no drug (reaction 2), 2.5 μM CPT (reaction 3), 160 μM SD8 (reaction 4), or both 2.5 μM CPT and 160 μM SD8 (reaction 5). After incubation at 37 °C for 10 min, SDS was added to 1%, and the reaction mixtures were incubated at 37 °C for 5 min. Then, EDTA and proteinase K were added to a final concentration of 25 mM and 100 μg/mL, respectively, followed by incubation at 37 °C for 15 min. A control reaction (reaction 1) was stopped by the addition of SDS (to a final concentration of 1%) after the 5 min incubation, and then dilution buffer was added to process it in the same manner as other reaction mixtures. The DNA products were purified and analyzed as described in the DNA cleavage assay for the Topo I section.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Fermentation, isolation, and purification procedures for SD8 are available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

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