DNA Polymerase and Topoisomerase II Inhibitors from *Psoralea corylifolia*

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An ethanol extract of *Psoralea corylifolia* caused strong DNA polymerase inhibition in a whole cell bioassay specific for inhibitors of DNA replication enzymes. Bioassay-directed purification of the active compounds led to the isolation of the new compound corylifolin (**1**) and the known compound bakuchiol (**2**) as DNA polymerase inhibitors. On the basis of the structures of **1** and **2**, resveratrol (**3**) was tested and found to be active as a DNA polymerase inhibitor in this bioassay. Neobavaisoflavone (**4**) was isolated as a DNA polymerase inhibitor, daidzein (**5**) as a DNA polymerase and topoisomerase II inhibitor, and bakuchicin (**6**) as a topoisomerase II inhibitor.

Psoralea corylifolia L. (Leguminosae) has been used to treat a wide variety of diseases and conditions in both Chinese and Indian folkloric medicine.[1−4] The essential oil of the plant is active at high dilution against *Streptococcus* and *Paramecium*.[3] The meroterpene, bakuchiol (**2**, Chart 1), was identified as an active constituent of the essential oil.[5] Bakuchiol has also been reported to have inhibitory activity against *Staphylococcus aureus*,[6−8] to inhibit weakly phospholipase 2A and modulate inflammatory responses,[9] and to have antitumor,[10] antimutagenic,[11] and insect hormonal[12] activities. Since the folkloric uses of *P. corylifolia* include use as an antineoplastic, we tested an ethanol extract of this plant with a Simian virus 40 (SV40)-based intact cell bioassay that can detect inhibitors of specific enzymes of mammalian DNA replication. Drugs that target enzymes of mammalian DNA replication are potentially useful for cancer chemotherapy or as probes for understanding the roles of specific enzymes in DNA replication and repair. An ethanol extract of *P. corylifolia* caused an unusually strong DNA polymerase inhibition signal in the SV40 bioassay, and the assay was then used to guide the purification of the active compounds. A new compound, corylifolin (**1**), was identified as a weak inhibitor of replicative DNA polymerases using the SV40 bioassay, and bakuchiol (**2**) was identified as a substantially stronger DNA polymerase inhibitor. On the basis of the structures of **1** and **2**, resveratrol (**3**, Sigma Chemical Co.) was tested and identified as a DNA polymerase inhibitor in the SV40 assay. Neobavaisoflavone (**4**) inhibited replicative DNA polymerases significantly at high concentrations, and daidzein (**5**) caused slight inhibition of both DNA polymerase and topoisomerase II at high concentrations. Bakuchicin (**6**) inhibited topoisomerase II in SV40-infected cells at high concentrations and had no effect on DNA polymerase. In an in vitro assay, **6** inhibited purified topoisomerase II with an IC_{50} of 404 μM, confirming that it is a weak topoisomerase II inhibitor. No topoisomerase II-DNA cross-links were detected, indicating that **6** is not a topoisomerase II poison. The bakuchicin analogue psoralen (**7**) was also isolated from *P. corylifolia* but was completely inactive in the bioassay.

Results and Discussion

HREIMS of corylifolin (**1**) gave a molecular ion peak at *m/z* 188.1173 (1) corresponding to molecular formula $C_{13}H_{16}O$. The ^{1}H NMR spectrum (CD_3COCD_3) of **1** showed the presence of two quaternary methyl (two 3H singlets at δ 1.17 and 1.09, H-12, H-13). A two-proton multiplet at δ 5.00 (2H, m, H-11) and a one-proton doublet doublet at δ 5.90 (1H, dd, J = 10.7, 17.4 Hz, H-10) could be assigned to an ABC pattern (−CH═CH_2). The two-proton doublet at δ 6.08 (1H, d, J = 16.3 Hz, H-8) and 6.27 (1H, d, J = 16.3 Hz, H-7) was assigned to a trans double bond (−CH═CH−). Four proton signals, essentially in a AA′BB′ quartet at δ 6.76 (2H, d, J = 8.6 Hz, H-3, H-5) and δ 7.23 (2H, d, J = 8.6 Hz, H-2, H-6), were assigned to four aromatic protons (*p*-disubstituted benzene ring). The singlet at δ 9.65 (1H, br s) could be assigned to the phenolic hydroxyl group. ^{13}C NMR (CD_3COCD_3) signals were observed at δ 153.1 (C-4), 147.3 (C-10), 135.6 (C-7), 131.8 (C-1), 128.0 (C-6), 128.0 (C-2), 127.6 (C-8), 115.8 (C-5), 115.8 (C-3), 111.9 (C-11), 43.0 (C-9), 25.0 (C-12), 23.9 (C-13). The ^{1}H and ^{13}C NMR data of **1** were assigned using a 2D-HETCOR experiment. Comparison of the ^{1}H NMR and ^{13}C NMR resonances of bakuchiol (**2**) and its analogues[13] indicated that they are all closely related and have carbon skeletons similar to **1**. Thus, the structure of corylifolin (**1**) was established as 1-(9,9-dimethyl-7(*E*),-10-pentadienyl)phenol. Mass spectral fragmentation (Figure 1) also supported this conclusion.

Compounds **1** and **2** caused accumulation of aberrant 40S viral replication intermediates in SV40-infected cells, a signature of replicative DNA polymerase inhibition in SV40-infected cells[21,22] (Figure 2). Assays with purified mammalian DNA polymerases showed that **2** inhibited DNA polymerase ϵ, one of the three "α-family"

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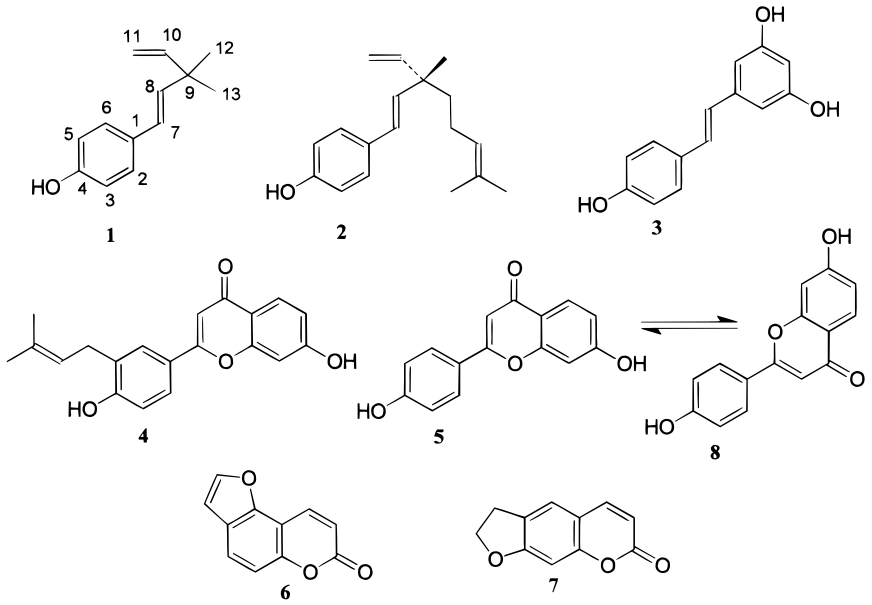
Chart 1



DNA polymerases involved in mammalian nuclear DNA replication, but had no effect on DNA polymerases β or γ, which are involved in DNA repair and mitochondrial DNA synthesis (Linn, S., University of California at Berkeley, unpublished data). This result suggests that **2**, like aphidicolin, causes 40S intermediates by direct inhibition of replicative DNA polymerases rather than by indirect mechanisms such as depletion of nucleotide pools. Since compounds **1** and **2** share a 4-hydroxystyryl moiety, resveratrol (**3**) was also tested in the bioassay and was found to be a DNA polymerase inhibitor (Figure 2). This suggests that the 4-hydroxystyryl moiety is a pharmacophore associated with activity as a DNA polymerase inhibitor. However, the relative weakness of **1** compared to **2** and **3** suggests that the groups associated with the 4-hydroxystyryl moiety modulate the activity strongly. In **2**, the carbon skeleton of **1** has been extended by the replacement of a methyl hydrogen at C-13 with an isopentenyl group. In **3**, the 9,9-dimethyl-7(*E*),10-pentadienyl group of **1** has been replaced by a 3,5-dihydroxyphenyl group. These groups in **2** and **3** may interact with a hydrophobic pocket on DNA polymerase or may facilitate transport across the cell membrane.

Neobavaisoflavone (**4**) caused inhibition of DNA polymerase at moderate to high concentrations, and daidzein (**5**) caused slight inhibition of both topoisomerase II and DNA polymerase at high concentrations (Figure 3). Flavones are known inhibitors of both DNA polymerase and topoisomerase II.[14] These flavones have some shared structural features with **1**−**3**. The 4-hydroxystyryl moiety associated with DNA polymerase inhibition in **1**−**3** is coincident with the phenolic B ring and the double bond in the A ring of **4** and **5**. In addition, **8**, a conformer of **5**, has an overall shape similar to that of **3**. Since free rotation is expected around the bond between the A and B rings of **5**, it is assumed that **5** and **8** are in equilibrium and raises the possibility that **8** may be the active conformer on the basis of its similarity to **3**. Neobavaisoflavone (**4**) did not significantly inhibit topoisomerase II in the bioassay and is a somewhat stronger DNA polymerase inhibitor than **5** in the assay. Since the only difference between **4** and **5** is the isopentenyl group at the C-5′ position of the latter compound, it seems likely that the presence of this group abolishes inhibition of topoisomerase II while increasing inhibition of DNA polymerase.

Bakuchicin (**6**) caused weak inhibition of topoisomerase II at high concentrations in the SV40 assay (Figure 3). In an in vitro assay with purified topoisomerase II, **6** inhibited the topoisomerase II reaction with an IC_{50} of 404 μM, confirming that it is a very weak topoisomerase II inhibitor. No topoisomerase II−DNA cross-links were detected in either the SV40 assay or in the in vitro assays, indicating that **6** is a catalytic topoisomerase II inhibitor but not a topoisomerase II poison.

Although daidzein (**5**) and bakuchicin (**6**) are weak topoisomerase II inhibitors, no topoisomerase II inhibition was detected at the crude ethanol extract level. These activities only became apparent during the late stages of fractionation. The SV40 assay can detect topoisomerase II inhibitors at the crude extract level and guide their purification,[22] but very weak active compounds such as **5** and **6** would only be expected to cause signals at the crude extract level if they were present in very high concentrations. The strong DNA polymerase inhibition caused by the crude extract appears to be mainly due to bakuchiol, which is the strongest of the DNA polymerase inhibitors found in *P. corylifolia* and which also appears to be present in the highest concentration. Corylifolin, daidzein, and neobavaisoflavone may contribute slightly to the DNA polymerase inhibition caused by the crude extract.

These results demonstrate that the SV40 bioassay can detect and guide the purification and isolation of inhibitors of DNA polymerases and topoisomerases. This approach can discover both new active compounds and new activities of known compounds of natural origin.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz,

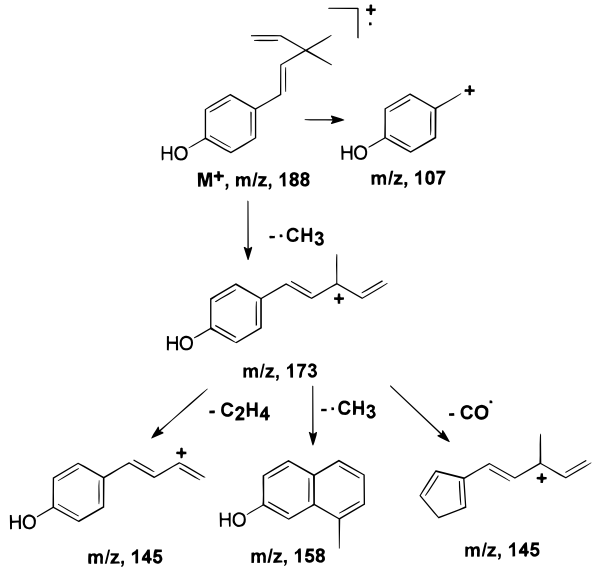


Figure 1. EIMS Cleavage of **1**.

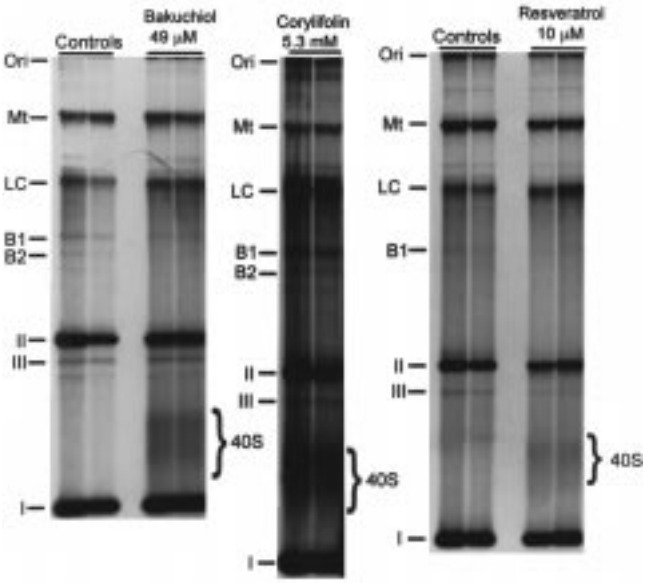


Figure 2. DNA polymerase inhibition by **1**−**3** in SV40-infected monkey kidney cells. The image is a fluorographic exposure on X-ray film of tritium pulse-labeled SV40 replication intermediates separated by agarose gel electrophoresis. Key: Ori, origin of electrophoresis; Mt, mitochondrial DNA; LC, late Cairns structure (late viral replication intermediates in which the terminal 5% of the viral chromosome remains to be replicated); B1, B2, catenated SV40 dimers (newly replicated but topologically linked daughter chromosomes in which one daughter chromosome is nicked and one is superhelical and the catenation linking number is indicated); II, form II (nicked circular) SV40 DNA; III, form III (double strand linear) SV40 DNA; I, form I (superhelical circular) SV40 DNA; 40S, torsionally stressed, compacted replication intermediates resulting from inhibition of DNA polymerase. Normal replication intermediates produce the even density between the form I and LC bands as seen in the controls. Controls include the solvent (DMSO).

respectively, on a Bruker AMX-500 spectrophotometer in $CDCl_3$ or CD_3COCD_3, using the solvent signal as internal standard. HREIMS were obtained on a VG 70-250S mass spectrometer. The UV spectrum was recorded with a Kontron Uvikon 860 spectrophotometer. The IR spectrum was obtained on an FX80, KVB/

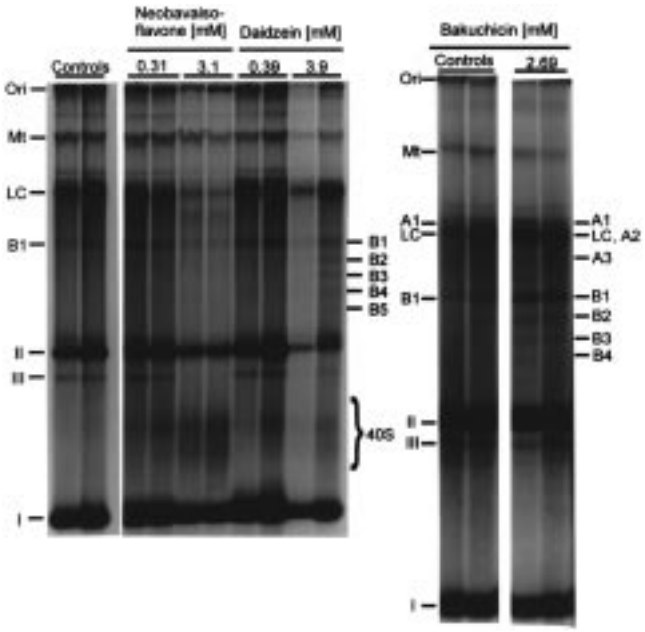


Figure 3. Inhibition of DNA polymerase and topoisomerase II in SV40-infected cells. Key: A1−A5, catenated (topologically linked) SV40 daughter chromosomes in which both daughter chromosomes are nicked and the catenation linking number is indicated; B1−B5, catenated SV40 daughter chromosomes in which one daughter chromosome is nicked and one is superhelical and the linking number is indicated. Other abbreviations are the same as in Figure 2. The 40S intermediates indicate DNA polymerase inhibition by **4** and **5**, and the increases in A- and B-family catenated dimer bands indicate topoisomerase II inhibition by **5** and **6**.

Analect spectrophotometer. The optical rotation was obtained on a Perkin-Elmer polarimeter. Column chromatography and preparative TLC were performed using Merck silica gel 60 powder and precoated Merck silica gel 60 F_{254} plates.

Plant Material. Fruits of *P. corylifolia* L. were purchased from Lin Sister Herb Shop, New York, NY. The plant is grown in Shang Qiu, Hunan province, China, and was collected and dried in September 1996. The voucher specimen is stored in the Ohio State University plant collection.

Extraction and Isolation. *P. corylifolia* fruit powder (300 g) was extracted with 95% EtOH to obtain 47.45 g of ethanol extract (F001). Of this, 46.84 g was loaded on a silica gel column that was eluted with acetone and MeOH to give 38.11 g of an acetone-soluble fraction (AC) and 8.45 g of a methanol-soluble fraction (ME). Strong DNA polymerase inhibiting activity was detected in the AC fraction using the SV40 bioassay. The AC fraction (34 g) was chromatographed on a silica gel column that was eluted with hexane and increasing concentrations of ethyl acetate (10−40%) in hexane to yield seven fractions, A−G. Repeated separation by column chromatography and preparative TLC of fractions B, D, and F resulted in isolation and purification of five active compounds: corylifolin (**1**, 63.1 mg), bakuchiol (**2**, 0.59 g), neobavaisoflavone (**4**, 153.7 mg), daidzein (**5**, 30 mg), and bakuchicin (**6**, 1.51 g). The inactive bakuchicin analogue psoralen (**7**, 2.96 g) was also isolated.

Corylifolin (1): amorphous; $[\alpha]^{20}_D$ +60° (*c* 0.07, MeOH); UV (MeOH) λ_{max} 261 nm; IR (KBr) ν_{max} 3284

(OH), 970 (C═C), 2973, 2933, 1623, 1610, 1513, 1459, 1375, 1267, 1241, 1193, 1170, 1099, 1070 cm^{-1}; HREIMS of compound **1** gave a molecular ion peak at *m/z* 188.173 (1) corresponding to molecular formula $C_{13}H_{16}O$ (calcd 188.1201), 173.0967 $[C_{12}H_{13}O]$ $[M - CH_3]^+$ (100) (calcd 173.0966, 158.0711 $[C_{11}H_{10}O]$ $[M - 2CH_3]^+$ (14) (calcd 158.0715), 145.1035 $[C_{11}H_{13}]$ $[M - CH_3CO]^+$ (1) (calcd 145.1017), 145.0653 $[C_{10}H_9O]$ $[M - CH_3{-}C_2H_4]^+$ (23) (calcd 145.0653), 107.0487 $[C_7H_7O]$ $[M - C_6H_9]^+$ (52) (calcd 107.0496), 77.0409 $[C_6H_5]$ $[M - C_7H_{11}O]^+$ (18) (calcd 77.0391); ^1H NMR (CD_3COCD_3) δ 9.65 (1H, br, s OH), 7.23 (2H, d, J = 8.6 Hz, H-2, H-6), 6.76 (2H, d, J = 8.6 Hz, H-3, H-5), 6.27 (1H, d, J = 16.3 Hz, H-7), 6.08 (1H, d, J = 16.3 Hz, H-8), 5.90 (1H, dd, J = 10.7, 17.4 Hz, H-10), 5.02 (2H, m, H-11), 1.17 (3H, s, H-12), 1.09 (3H, s, H-13); ^{13}C NMR (CD_3COCD_3) δ 153.1 (C-4), 147.3 (C-10), 135.6 (C-7), 131.8 (C-1), 128.0 (C-6), 128.0 (C-2), 127.6 (C-8), 115.8 (C-5), 115.8 (C-3), 111.9 (C-11), 43.0 (C-9), 25.0 (C-12), 23.9 (C-13).

Bakuchiol (2). HREIMS of compound **2** gave a molecular ion peak at *m/z* 256.1835 (13) corresponding to the molecular formula $C_{18}H_{24}O$ (calcd 256.1827). It was identified as bakuchiol by comparison of MS and NMR spectra with literature data.[5,13,15,16]

Neobavaisoflavone (4). HREIMS of compound **4** gave a molecular ion peak at *m/z* 322.1186 (100) corresponding to the molecular formula $C_{20}H_{18}O_4$ (calcd 322.1204). Compound **4** was identified as neobavaisoflavone by comparison of MS and NMR spectra with literature data.[17]

Daidzein (5). HREIMS of compound **5** gave a molecular ion peak at *m/z* 254.0585 (100) corresponding to the molecular formula $C_{15}H_{10}O_4$ (calcd 254.0579). Compound **5** was identified as daidzein by comparison of MS and NMR spectra with literature data.[18,19]

Bakuchicin (6). HREIMS of compound **6** gave a molecular ion peak at *m/z* 186.0344 (100) corresponding to the molecular formula $C_{11}H_6O_3$ (calcd 186.0316). Compound **6** was identified as bakuchicin by comparison of MS and NMR spectra with literature data.[20]

SV40 Bioassay. Simian virus 40 infected CV-1 monkey kidney cells were used to detect drugs that interfere with specific enzymes of DNA replication.[21,22] Briefly, viral DNA replication intermediates, which make extensive use of host cell enzymes of DNA replication, were pulse-labeled with ^3H-Tdr for 30 min, and extracts, fractions, or pure compounds to be tested were added 15 min after the start of the pulse label. After a 15 min exposure, the labeling reaction was terminated by addition of Hirt lysis solution, which stops DNA synthesis by lysing the cells and denaturing proteins and enzymes. The labeled viral chromosomes were selectively extracted and analyzed by high-resolution agarose gel electrophoresis as described.[23] SV40, a double-stranded, circular DNA virus, is grown in monkey cells and makes such extensive use of host cell enzymes of DNA replication and chromatin that it is considered a "minichromosome" or model for the mammalian replicon.[24] In this capacity, the virus has been used to understand the molecular events leading to lethal chromosome damage from topoisomerase inhibitors and DNA polymerase inhibitors.[21] The actively replicating viral chromosomes can be pulse-labeled with tritiated thymidine, selectively extracted, and analyzed by agarose gel electrophoresis and gel fluorography. Specific DNA-damaging agents and specific inhibitors of different enzymes of DNA replication produce unique aberrant viral replication intermediates that appear as signature patterns of bands in gel electrophoretic separations of viral replication intermediates. The patterns are diagnostic for the type of DNA-damaging agent or enzyme inhibitor present and can be used to detect and purify active compounds from crude extracts of plants.[22] Inhibition of DNA polymerases involved in replication of nuclear DNA causes the rapid accumulation of torsionally stressed 40S replication intermediates in SV40-infected cells.[25,26] These 40S intermediates migrate as a distinct broad band just behind the form I (superhelical circle) viral DNA on one-dimensional agarose gel electrophoresis and form very unique patterns on two-dimensional electroporetograms.[26] The identity of 40S intermediates detected by one-dimensional gel electrophoresis was confirmed by two-dimensional gel experiments and other criteria (data not shown). Inhibition of topoisomerase II causes the accumulation of highly catenated (topologically linked) daughter SV40 chromosomes that appear as distinct overlapping ladders of bands in gel electrophoretic separations of viral replication intermediates.[27,28]

Assay for Topoisomerase II Inhibition in Vitro. The assay for catalytic inhibition of topoisomerase II measures decatenation of highly catenated kinetoplast DNA (kDNA) networks by purified topoisomerase II.[29,30] The total reaction volume of 20 μL included decatenation assay buffer (50 mM Tris−HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT, 30 μg BSA/mL) and kDNA (100 ng/reaction). The amount of topoisomerase II was adjusted to give 80−90% decatenation in 30 min at 37 °C. Reactions were initiated by addition of the topoisomerase II. Reactions were terminated by the addition of 1/5 volume of stop buffer/loading dye (5% Sarkosyl, 0.0025% bromophenol blue, 25% glycerol). The samples were electrophoresed in 1% agarose submarine gels with 0.5 μg/mL ethidium bromide in the TAE electrophoresis buffer (40 mM Tris acetate, pH 8.0, 1 mM EDTA). The bands of kDNA and free DNA circles were visualized by UV trans-illumination and were quantitated with an IX-1000 digital imaging system (Alpha Innotech Corp., San Leandro, CA). Topoisomerase II and kDNA were generous gifts of TopoGen, Columbus, OH.

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