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Inhibition of Topoisomerase II by ICRF-193, the Meso Isomer of 2,3-Bis(2,6-dioxopiperazin-4-yl)butane

CRITICAL DEPENDENCE ON 2,3-BUTANEDIYL LINKER ABSOLUTE CONFIGURATION

Robert M. Snapka,† Sung Ho Woo,* Andrei V. Blokhin‡ and Donald T. Witiak‡*

*Departments of Radiology and Medical Microbiology & Immunology, The Ohio State University, Columbus, OH 43210; and ‡Division of Medicinal Chemistry, School of Pharmacy, University of Wisconsin-Madison, Madison, WI 53706-1515, U.S.A.

ABSTRACT. The bis(2,6-dioxopiperazine)s are a structurally and mechanistically unique class of topoisomerase II inhibitors that do not bind DNA and that do not stabilize topoisomerase II-DNA strand passing intermediates ("cleavable complexes"). The most effective topoisomerase II inhibitor in the bis(2,6-dioxopiperazine) series is ICRF-193 (meso or *S**, *R** isomer), with a meso 2,3-butanediyl linker connecting the dioxopiperazine rings. The two enantiomeric diastereomers, (*R,R*) and (*S,S*), of ICRF-193 possessing the two optically active 2,3-butanediyl linkers have been prepared from their respective optically pure 2,4-diaminobutanes via 2,3-diaminobutane-*N,N,N′,N′*-tetraacetic acid, esterification, and imide formation. Both *in vivo* and *in vitro* assays for catalytic inhibition of topoisomerase II were employed to show that the (*S,S*)- and (*R,R*)-isomers are almost inactive as topoisomerase II inhibitors. The data indicate that the meso stereochemistry of the alkanediyl linker is crucial for activity and provides additional evidence that the cytotoxicity of the bis(2,6-dioxopiperazine)s is due to their ability to inhibit topoisomerase II. BIOCHEM PHARMACOL 52;4:543–549, 1996.

KEY WORDS. ICRF-193; bis(dioxopiperazine); topoisomerase II; inhibitor; antagonist

DNA topoisomerases are essential enzymes of mammalian cells. Both type I and type II topoisomerases can release superhelical stress in DNA resulting from transcription or DNA replication [1], and type II topoisomerases are required for the separation of topologically linked daughter chromosomes following DNA replication (reviewed in Ref. 2). Many antineoplastic drugs target topoisomerases by stabilizing an intermediate in the DNA strand passing reaction. Drugs that stabilize these "cleavable complexes" are known as topoisomerase poisons, and include well known anticancer agents such as Adriamycin® and m-AMSA§ (topoisomerase II poisons) and camptothecin (a topoisomerase I poison) [3]. Recently, drugs that inhibit mammalian topoisomerase II (EC 5.99.1.3) without stabilizing cleavable complexes have been reported to have anticancer activity [4–6]. These drugs are sometimes known as topoisomerase antagonists or catalytic inhibitors [7]. The bis(2,6-dioxopiperazine) topoisomerase II antagonists interact directly with topoisomerase II [8] rather than with DNA or with a topoisomerase-DNA complex. The bis(dioxopiperazine)s (Fig. 1) are structurally unique, and bear no resemblance to other known topoisomerase II inhibitors. It is likely that their interaction with topoisomerase II is also unique and that these drugs have opened a new avenue for the development of anticancer agents.

The biologically active bis(2,6-dioxopiperazine)s are antitumor and antimetastatic agents that can inhibit DNA synthesis. Structure-activity relationships for the anticancer activity of bis(2,6-dioxopiperazine)s and their interactions with other anticancer drugs have been reviewed [9]. The intracellular target or mechanism of action was not originally known, but it was assumed to involve binding of metal ions. The bis(2,6-dioxopiperazine)s were designed as membrane permeable analogs of the metal chelator EDTA [10]. The parent compound ICRF-154 (**1d**) has significant anticancer activity in animal tumor models, and its analog ICRF-159 (Razoxane, with a 1,2-propanediyl linkage of the dioxopiperazine rings, **1e**) has similar activity in these same systems [9]. With the discovery that these drugs target mammalian topoisomerase II [4], structure–activity studies focused on topoisomerase II have begun. The most efficient

† Corresponding author: Dr. Robert M. Snapka, Department of Radiology, Division of Radiobiology, The Ohio State University, 103 Wiseman Hall, 400 West 12th Ave., Columbus, OH 43210. Tel. (614) 292-9375; FAX (614) 292-7237.

§ *Abbreviations:* m-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; VM-26 (teniposide), [4′-dimethylepipodophyllotoxin 9-(4,6-*O*-2-thenylidene-β-D-glucopyranoside); Tdr, thymidine; kDNA, kinetoplast DNA; PMP, 1,2,2,6,6-pentamethylpiperidine; MsCl, methanesulfonyl chloride; DMF, dimethylformamide, MeOH, methanol; EtOAc ethyl acetate; Et_2O, diethyl ether; and Pd/C, palladium/carbon.

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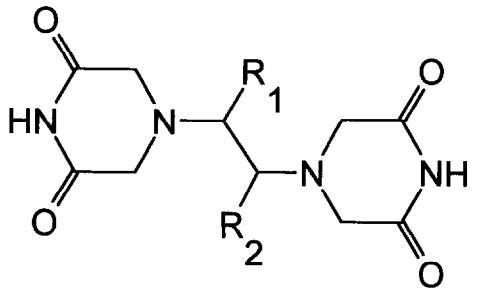


1a. R_1 = *(R)* Me; R_2 = *(R)* Me, **(R,R) isomer of ICRF-193**
1b. R_1 = *(S)* Me; R_2 = *(S)* Me, **(S,S) isomer of ICRF-193**
1c. R_1 = *(R)* Me; R_2 = *(S)* Me, **ICRF-193 (meso)**
1d. R_1 = H; R_2 = H, **ICRF-154**
1e. R_1 = H; R_2 = Me, **ICRF-159 (racemic)**
1f. R_1 = H; R_2 = *(S)* Me, **ICRF-186**
1g. R_1 = H; R_2 = *(R)* Me, **ICRF-187**
1h. R_1 = Me; R_2 = Et, **ICRF-202 (racemic, erythro)**
1i. R1 = *(R)* Et; R2 = *(S)* Et, **ICRF-201 (meso)**
1j R1 = H; R2 = Et, **ICRF-192 (racemic)**

FIG. 1. Bis(2,6-dioxopiperazine)s.

inhibitor of topoisomerase II studied to date is the meso 2,3-butanediyl analog ICRF-193 (**1c**) [4, 11]. In this study, we have prepared the optically pure (*R,R*)-**1a** and (*S,S*)-**1b** diastereomers of ICRF-193 and show that they are almost inactive as topoisomerase II inhibitors. This shows that the meso stereochemistry is essential for efficient inhibition of topoisomerase II.

MATERIALS AND METHODS

Synthesis of 2,3-Bis(2,6-dioxopiperazin-4-yl)butanes of Known Absolute Configuration

(*R,R*)-2,3-Butanediol was purchased from Fluka (Ronkonkoma, NY); other chemicals were purchased from Aldrich (Milwaukee, WI) and used as received. Melting points were determined in open capillaries with a Thomas–Hoover Uni-Melt Apparatus and are uncorrected. NMR spectra were obtained with a Bruker AM 300 spectrometer. Chemical shifts are reported on the δ scale with peak multiplicities: s, singlet; d, doublet; dd, doublet of doublets; and m, multiplet; *J* values are reported in Hz. IR spectra were obtained in KBr with a Perkin–Elmer 599B spectrophotometer and are reported in cm^{-1}. Optical rotations were taken on a Perkin–Elmer model 241 polarimeter using a 1-mL 10-cm cell. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.

General Procedure for Hydrogenation of Diazides 4a–c; 2(R),3(S)-Diaminobutane 5c

A mixture of diazide **4c** (15 g, 107 mmol), 10% Pd/C (500 mg) in 200 mL of methanol was stirred under H_2 (30 psi) for 24 hr. The catalyst was filtered (Celite), and the filtrate was evaporated under Ar. Fractional distillation afforded diamine **5c** (7.53 g, 80%) with b.p. 57–60°/60 mm Hg. (Lit b.p. [12]: 58.8–61.8°/60 mm Hg.)

Preparation of 2(R),3(R)- and 2(S),3(S)-Diaminobutanes 5a and b from Diazides 4a and b

The 2(*R*),3(*R*)- and 2(*S*),3(*S*)-diaminobutanes **5a** and **b** were prepared from diazides **4a** and **b** (80–85% yields). Enantiomers **5a** and **b** displayed $[\alpha]_D^{20}$ −29.42 and +29.41 (*c* 2.4, benzene) [**5b** lit. $[\alpha]_D$ +29.40 [13] (*c* 2.4, benzene)].

General Procedure for the Preparation of Tetramethyl 2,3-Diaminobutane-N,N,N′,N′-tetraacetates 6a–c; (R,R)-Isomer 6a

Bromoacetic acid (37.25 g, 268 mmol) was dissolved in 64 mL of H_2O and neutralized with aqueous NaOH solution (10.72 g, 268 mmol in 64 mL of H_2O) at 0°. Under Ar, diamine **5a** (3.93 g, 44.7 mmol) and NaOH solution (10.72 g, 262 mmol in 64 mL of H_2O) were added with stirring, and after 10 hr, at 20° the reaction mixture was heated at 45° for 2 hr and acidified to pH 1.5 with concentrated HCl. After H_2O was removed *in vacuo*, the residue was suspended in 2,2-dimethoxypropane (983 mL) and concentrated HCl (45 mL). The reaction mixture was stirred for 12 hr, all volatiles were removed *in vacuo*, and the residue was poured into 450 mL of ice-cold H_2O. The aqueous solution was extracted with $CHCl_3$ (50 mL × 4), then neutralized with $NaHCO_3$, and extracted with EtOAc (70 mL × 4) to yield 11.28 g (67%) of product **6a.** Distillation with a Kugelrohr apparatus (158°/0.07 mm) afforded an analytical pure product as a light yellow oil. $[\alpha]_D^{20}$ −25.13 (*c* 1.6 $CHCl_3$); ^1H NMR ($CDCl_3$): 1.04 (d, *J* = 6.3, 6H, CH_3), 2.80–2.86 (m, 2H, CH), 3.54 (d, *J* = 17.5, 4H, CH_2), 3.68 (d, *J* = 17.4, 4H, CH_2), 3.68 (s, 12H, CH_3O); ^{13}C NMR ($CDCl_3$): 13.4 (CH_3), 51.3 (CH_3O), 52.6 (CH_2), 60.2 (CH), 172.6 (CO); IR: 2980, 1770, 1750, 1445, 1210. Anal. calc. for $C_{16}H_{28}O_8N_2$: C, 51.06; H, 7.45; N, 7.45. Found: C, 50.77; H, 7.23; N, 7.21.

Preparation of the (S,S)-Isomer 6b from the Diamine 5b

The (*S,S*)-isomer **6b** was prepared from the diamine **5b** (4.00 g, 45.5 mmol) with a yield of 12 g (70%). $[\alpha]_D^{20}$ +25.11 (*c* 1.6, $CHCl_3$); ^1H and ^{13}C NMR and IR results were identical to those of enantiomer **6a.**

Preparation of the (R,S)-Isomer 6c from the Diamine 5c

The (*R,S*)-isomer **6c** was prepared from diamine **5c** (5.5 g, 62.5 mmol). After neutralization of the H_2O layer with $NaHCO_3$ solution the product was filtered and dried *in vacuo* to yield 16.1 g (74%) of **5c.** An analytical sample was obtained by recrystallization from hexane–EtOAc, m.p. 77–78°. ^1H NMR ($CDCl_3$): 1.12 (d, *J* = 6.1, 6H, CH_3), 2.62–

2.75 (m, CH, CH_3), 3.50 (s, 8H, CH_2), 3.68 (s, 12H, CH_3O); ^{13}C NMR ($CDCl_3$): 12.4 (CH_3), 51.4 (CH_3O), 52.5 (CH_2), 61.9 (CH), 172.5 (CO); IR: 3020, 2980, 1780, 1755, 1440, 1210. Anal. calc. for $C_{16}H_{28}O_8N_2$: C, 51.06; H, 7.45; N, 7.45. Found: C, 50.81; H, 7.25; N, 7.23.

(R,S)-2,3-Diaminobutane-N,N,N',N'-tetraacetamide 7

The tetraester **6c** (0.4 g, 1.1 mmol) and 28 mL of 2.0 M ammonia solution in methanol were heated in a sealed tube at 150° for 24 hr. The precipitate was filtered and recrystallized from H_2O to yield 0.13 g (39%) of a product, m.p. 280° (decomp.). ^1H NMR (DMSO-d_6): 1.03 (d, J = 4.5, 6H, CH_3), 2.25–2.40 (m, 2H, CH), 2.95 (s, 8H, CH_2), 7.11 (s, 2H, NH_2), 7.75 (s, 2H, NH_2); IR: 3470, 3400, 3230, 3190, 2980, 2950, 1680, 1660, 1430, 1350, 1130. Anal. calc. for $C_{12}H_{24}N_6O_4 \cdot 0.25\ H_2O$: C, 44.92; H, 7.64; N, 26.21. Found: C, 45.19; H, 7.88; N, 26.00.

General Procedure for the Preparation of 2,3-Bis (2,6-dioxopiperazin-4-yl)butanes 1*a–c; (R,R)-Isomer* 1*a*

A mixture of **6a** (1.88 g, 5 mmol) and formamide (2.25 g, 50 mmol) in 25 mL of anhydrous 1,4-dioxane was added slowly to a stirred suspension of NaH (1.39 g, 58 mmol) in 45 mL of 1,4-dioxane under Ar at 20°. The reaction mixture was stirred for 36 hr, the solvent was evaporated *in vacuo* at 20°, and the residue was suspended in Et_2O and quenched with 5 g of ice. The resulting emulsion was diluted with 50 mL of water and neutralized with concentrated HCl solution to pH 7 followed by evaporation of H_2O *in vacuo* at 20°. The residue was separated on a silica gel using 6.5% MeOH in $CHCl_3$ as eluant to yield 0.76 g (52%) of the product **1a.** The product was recrystallized from MeOH to afford an analytical sample with m.p. 217° (decomp.). $[\alpha]_D^{20}$ +37.50 (c 0.71, MeOH); ^1H NMR (D_2O): 0.85 (d, J = 6.0, 6H, CH_3), 2.75–2.85 (m, 2H, CH), 3.32 (d, J = 17.6, 4H, CH_2), 3.46 (d, J = 17.5, 4H, CH_2); ^{13}C NMR (D_2O, ref. signal CH_3OH = 49.0): 11.7 (CH_3), 50.6 (CH_2), 58.9 (CH), 174.3 (CO); IR: 3310, 3080, 3000, 2980, 2820, 1720, 1325, 1295, 1270, 1180, 1130, 1000. Anal. calc. for $C_{12}H_{18}N_4O_4$: C, 51.06; H, 6.38; N, 19.86. Found: C, 50.85; H, 6.58; N, 19.67.

Preparation of (S,S)-Isomer 1*b from the Tetraester* 6*b*

The (S,S)-isomer **1b** was prepared from the tetraester **6b** (1.88 g, 5 mmol) with a yield of 0.82 g (57%). $[\alpha]_D^{20}$ −37.70 (c 1.09, MeOH). m.p., ^1H and ^{13}C NMR and IR results were identical with those reported for enantiomer **1a.**

Preparation of the (R,S)-Isomer 1*c from the Tetraester* 6*c*

The (*R,S*)-isomer **1c** was prepared from the tetraester **6c** (8 g, 21 mmol). After neutralization of the reaction mixture with concentrated HCl to pH 7, the solution was cooled in a refrigerator; the precipitate was filtered, washed with cold H_2O, acetone, and Et_2O, and dried *in vacuo* to afford 4.78 g (80%) of the product **1c,** m.p. 318° (decomp.). [Lit. [14] value 320° (decomp.).] ^1H NMR (DMSO-d_6): 0.88 (d, J = 5.3, 6H, CH_3), 2.70–2.85 (m, 2H, CH), 3.22 (d, J = 16.6, 4H, CH_2), 3.33 (d, J = 16.2, 4H, CH_2), 10.98 (s, 2H, NH). ^{13}C NMR (DMSO-d_6): 9.2 (CH_3), 51.7 (CH_2), 58.7 (CH), 172.1 (CO); IR: 3220, 3110, 3000, 2940, 2820, 1755, 1710, 1325, 1280, 1170, 1140, 1030.

Assays for Inhibition of Topoisomerase II

Two *in vivo* assays (with intact cells) and an *in vitro* assay (with purified topoisomerase II and a DNA substrate) were used to evaluate the activity of these stereoisomers as inhibitors of topoisomerase II. The (*R,R*)- and (*S,S*)-stereoisomers were assayed after being dissolved in deionized water and after being dissolved in DMSO. Solvent controls were included in the assays.

In Vivo *Assay for Inhibition of Topoisomerase II*

Inhibition of topoisomerase II in simian virus 40 (SV40)-infected CV-1 cells causes an increase in the levels of catenated SV40 daughter chromosomes [2, 15]. Drugs were added to the virus-infected cells during a pulse labeling of replicating viral DNA with [^{3}H]Tdr [15]. The cells were lysed, and viral chromosomes were selectively extracted, processed, and separated by electrophoresis as described [16]. The electrophoretically separated viral replication intermediates were visualized by gel fluorography.

In Vivo *Assay for Topoisomerase II Antagonists*

Catalytic inhibitors of topoisomerase II which do not stabilize the "cleavable complex" DNA strand passing intermediate typically can reduce the level of DNA cleavage caused by topoisomerase II alone and can prevent topoisomerase poisons from stabilizing cleavable complexes. Thus, they can be classed as topoisomerase antagonists [6, 7]. Blocking of VM-26-induced topoisomerase II-DNA cross-links to pulse-labeled SV40 DNA was assayed in these experiments. Infected cells were pulse-labeled with [^{3}H]Tdr (250 μCi/mL) at the peak of viral DNA replication (36 hr post-infection). Bis(2,6-dioxopiperazine)s were added to the infected cells during the pulse-labeling, and VM-26 was added 15 min later (final concentration 100 μM). In the absence of other drugs, this concentration of VM-26 causes approximately 55% of the pulse-labeled viral DNA to be cross-linked to protein (the topoisomerase II). Pre-exposure to topoisomerase II antagonists reduces this cross-linking in a dose-dependent manner [6, 7]. After 15 min of exposure to VM-26, the cells were lysed, and pulse-labeled SV40 chromosomes were selectively extracted and assayed for protein–DNA cross-links [7].

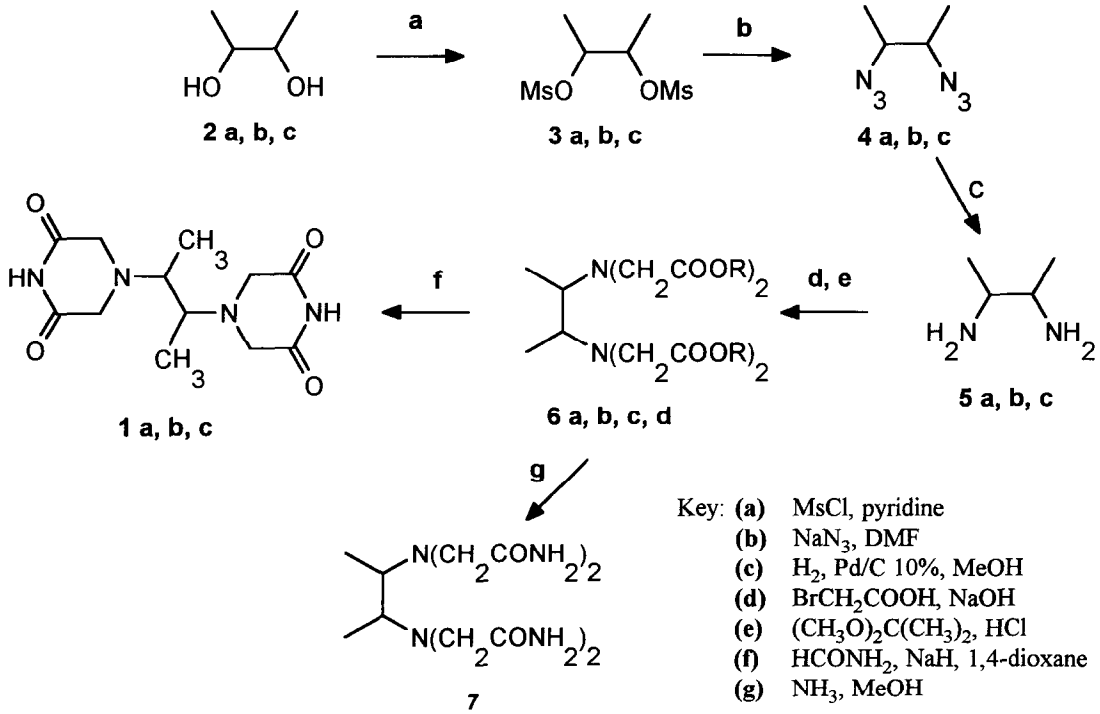
In Vitro *Assay for Inhibition of Topoisomerase II*

kDNA, recombinant human topoisomerase II, and other materials for the assay were a gift from TopoGEN, Inc. (Columbus, OH) [17]. kDNA is composed of DNA circles topologically linked to one another to form a large network. Topoisomerase II can decatenate the kinetoplast network by carrying out a double-strand DNA passing reaction to yield free DNA circles. The amount of topoisomerase II was adjusted to give complete decatenation in 30 min in a reaction mixture containing the solvent used to dissolve the test drug. Test drugs were dissolved immediately before use. ICRF-193 was dissolved in DMSO, and the (*R,R*)- and (*S,S*)-isomers were dissolved in both dH_2O and DMSO (for separate assays). Drug stocks were diluted so that the concentration of drug could be varied without changing the final concentration of the solvent in the topoisomerase II reaction mixture. Topoisomerase II reactions were stopped by addition of a buffer containing Sarkosyl, and the mixture was loaded directly on a 1% agarose submarine gel. The free DNA circles were separated from the substrate kDNA network by electrophoresis. The kDNA network is too large to enter the gel and remains at the edge of the loading well, whereas the free DNA circles produced by the topoisomerase reaction migrate as a band. The bands of kDNA and free DNA circles were visualized by staining with ethidium bromide and UV trans-illumination. The gel separations were photographed on Kodak Tmax 400 (4 × 5 in.) film, and reactions were quantitated by densitometric scanning of photographic negatives.

RESULTS

Synthesis of 2,3-Bis(2,6-dioxopiperazine-4-yl)butanes of Known Absolute Configuration

The preparation of optically pure 2,3-diaminobutanes was first achieved by resolution of racemic diamines using tartrates [12]. This procedure is not only time consuming, but provides sparing yields of diastereomers with insufficient purity even after 16 recrystallizations [18]. For these reasons, we made use of commercially available enantiomerically pure asymmetric diols **2** as starting materials (Fig. 2) [13]. The more expensive isomer **2a** was prepared from the diethyl ester of natural (*R,R*)-(+)-tartaric acid [19]. The conversion of the dimesilate **3** to the diazo derivative **4** proceeds with inversion of stereochemistry. Thus, (*R,R*)-diazide **4a** was obtained from (*S,S*)-dimesilate **3a**, and (*S,S*)-**4b** was prepared from the (*R,R*)-precursor **3b**. Transformation of diazides **4** to the diamines **5** by hydrogenation rather than $LiAlH_4$ reduction [18] provides for facile isolation and 80–85% yields. Hydrogenation can be monitored by NMR and proceeds without loss of compound due to adsorption by inorganic materials. The alkylation of di-



6 a, b, c, R = CH_3; **6d,** R = C_2H_5
2, 3 a, *(S,S)*-; **b,** *(R,R)*-; **c,** *(R,S)*-isomers
1, 4-6 a, *(R,R)*-; **b,** *(S,S)*-; **c, d,** *(R,S)*-isomers

FIG. 2. Synthetic scheme.

amine **5c** using ethyl bromoacetate in the presence of PMP and 1,4-dioxane [20] affords tetraethyl (*R,S*)-2,3-diaminobutane-*N,N,N',N'*-tetraacetate **6d** in 40% yield. Use of K_2CO_3 in DMSO [20] provides only 20% yields. Alternatively, use of bromoacetic acid in aqueous NaOH solution [14] is problematic because of the high product (2,3-diaminobutane-*N,N,N',N'*-tetraacetic acid) water solubility. However, concentration of the reaction mixture to dryness and esterification with 2,2-dimethoxypropane [21] affords tetraester **6c** in 74% isolated yield.

Enantiomeric tetramethyl esters **6a,b** are liquids, whereas the meso isomer **6c** is a solid (m.p. 77–78°). All tetraesters undergo reaction with formamide in the presence of NaH in 1,4-dioxane to yield the respective solid dioxopiperazines **1a–c,** but different methods were required for optimum yields. Use of the tetramethyl rather then the tetraethyl ester facilitates cyclization to 2,6-dioxopiperazines **1a–c.** The (*R,S*)-isomer **1c** is stable, but water-insoluble compound that precipitates following evaporation of the dioxane and quenching of the reaction mixture with acid. The enantiomers **1a** and **1b** are water soluble (10 mg/1 mL) and do not form a precipitate under the aforementioned conditions. Pure enantiomers were isolated following removal of H_2O *in vacuo* and purification of the residue over a silica gel column using $CHCl_3$ as eluant. Products **1a,b** are unstable in H_2O with a $T_{1/2}$ (NMR) of 24 hr. Tetraamide **7** is obtained by reaction of tetraester **6c** with 2 M ammonia solution in MeOH (sealed tube; 150°).

Inhibition of Topoisomerase II

ICRF-193 (meso, **1c**) caused a concentration-dependent increase in catenated SV40 daughter chromosomes (Fig. 3) when added to infected CV-1 cells. Distinct increases in B-family catenated dimers with lower catenation linking numbers were caused by 10 μM ICRF-193. At higher concentrations, the accumulations of very highly catenated B-family dimers (MC) and the fully superhelical C-family catenated dimers were evident. This is a typical pattern of increasing inhibition of topoisomerase II-dependent decatenation in SV40-infected cells [22]. As expected, this trend was accompanied by a concentration-dependent decrease in form I DNA, the product of the decatenation reaction. Enantiomers (*S,S*)-**1b** (Fig. 3) and (*R,R*)-**1a** (data not shown), diastereomers of meso ICRF-193 **(1c),** however, did not inhibit the topoisomerase II-dependent decatenation step of SV40 DNA replication. The (*S,S*)-**1b** result in Fig. 3 was done with the compound freshly dissolved in dH_2O. The same result was obtained with both diastereomers whether freshly dissolved in dH_2O or in DMSO (data not shown). The open ring form **7** was also inactive. ICRF-193 was also able to prevent VM-26 from stabilizing topoisomerase II–DNA complexes *in vivo*, as shown in Fig. 4. Thus, the meso isomer prevents topoisomerase II from carrying out its DNA strand passing reaction. The maximum inhibitory effect was achieved at about 20 μM ICRF-193. The (*S,S*)- and (*R,R*)-stereoisomers had no detectable effect.

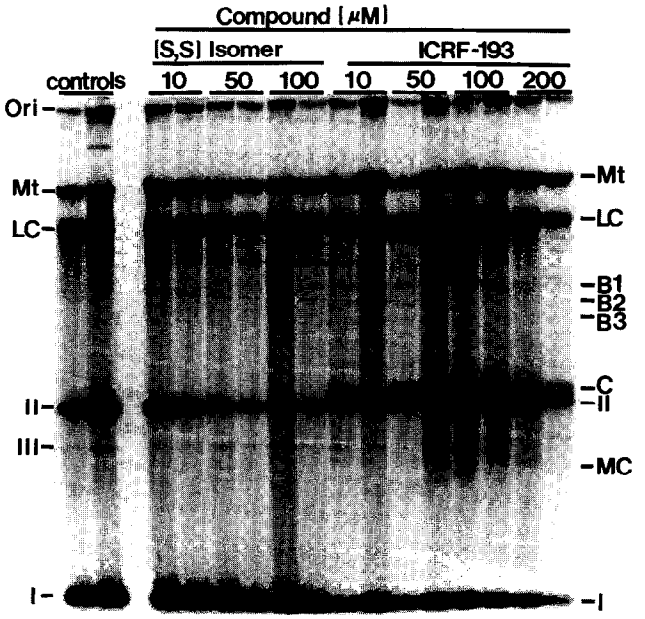


FIG. 3. Inhibition of topoisomerase II-dependent decatenation of newly replicated SV40 daughter chromosomes. The concentration–responses for ICRF-193 and the (*S,S*)-stereoisomer are shown. Untreated controls are shown in duplicate at the left. SV40-infected CV-1 cells were labeled for 30 min with [^{3}H]Tdr (250 μCi/mL) 36 hr after the start of the infection. Drugs were added to the labeling medium at the indicated concentrations 15 min after the start of the labeling, and the reactions were terminated by the addition of detergent-containing Hirt lysing fluid. Abbreviations: Ori, origin of electrophoresis (loading slot); Mt, mitochondrial DNA; LC, late Cairns structure (a figure "8" replication form with only the terminal 5% of the viral genome remaining to be replicated); II, form II (nicked circular) SV40 DNA; III, form III (double-strand linear SV40 DNA); I, form I (superhelical SV40 DNA); B1–B3, B-family catenated dimers (one daughter chromosome nicked and one superhelical) with the catenation linking number indicated; C, C-family catenated SV40 daughter chromosomes (both daughter chromosomes superhelical); MC, the point at which B-family catenated dimer bands are no longer resolved. Normal viral DNA replication intermediates are distributed as a continuous smear between the form I and LC bands. For reviews of SV40 DNA replication and the gel system, see Refs. 2 and 16.

Since the *in vivo* assays are insensitive to drugs that do not readily cross cell membranes, it was necessary to use an *in vitro* assay for topoisomerase II inhibition to confirm the lack of activity for the (*S,S*)- and (*R,R*)-stereoisomers. ICRF-193 caused a concentration-dependent inhibition of kDNA decatenation by purified recombinant human topoisomerase II (Fig. 5), but the (*S,S*)- and (*R,R*)-stereoisomers gave only marginal inhibition of decatenation at very high (millimolar) concentrations (DMSO solvent). No inhibitory activity was detected for the (*R,R*)- and (*S,S*)-diastereomers when they were dissolved in dH_2O just before assay at concentrations up to 100 μM (data not shown). The open ring form **(7)** was also inactive in the *in vitro* assay.

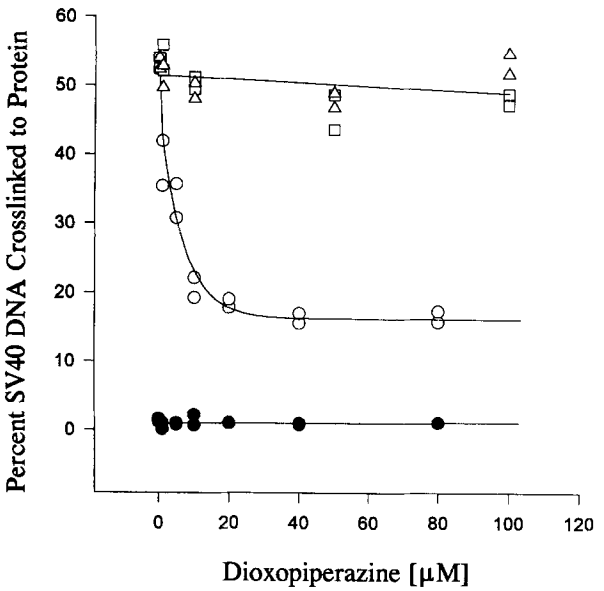


FIG. 4. Blocking of VM-26-induced topoisomerase II-DNA cross-links. SV40-infected CV-1 cells were labeled for 35 min with [^{3}H]Tdr (250 μCi/mL) 36 hr after the start of the infection. ICRF-193 and the (*R,R*)- and (*S,S*)-isomers were added to the labeling medium 15 min after the start of labeling, and VM-26 was added 5 min later (100 μM final concentration). The reactions were terminated by addition of Hirt lysing fluid 35 min after the start of labeling. Key: ICRF-193 (○); (*S,S*)-isomer (□); (*R,R*)-isomer (△); and ICRF-193 samples treated with proteinase K before assay for protein–DNA cross-links (●).

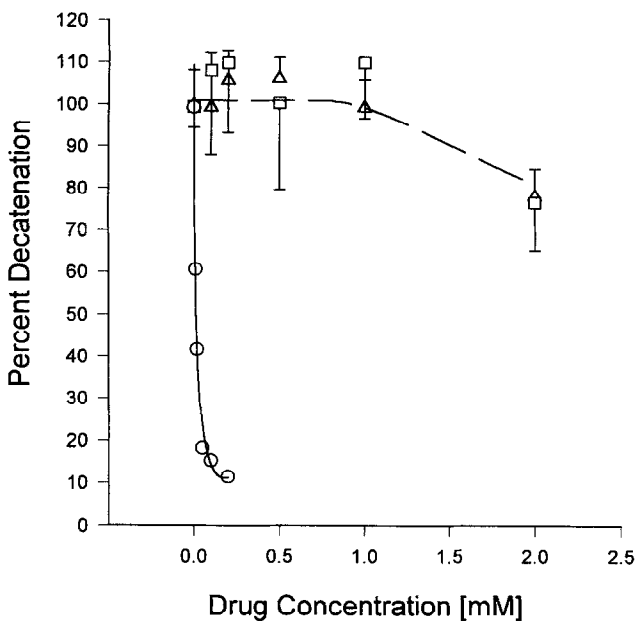


FIG. 5. Inhibition of topoisomerase II-dependent decatenation *in vitro*. Reaction mixtures (20 μL) contained 10 ng kDNA and 3 units of topoisomerase II per μL. Every reaction mixture contained 5% DMSO. Decatenation reactions were carried out for 15 min at 37°. Key: ICRF-193 (○); (*S,S*)-isomer (□); and (*R,R*)-isomer (△).

DISCUSSION

ICRF-193 (**1c**), the meso isomer of 2,3-bis(2,6-dioxopiperazine-4-yl)butane, is the most potent topoisomerase II inhibitor in the bis(dioxopiperazine) series [4, 11]. With respect to ICRF-193, all reported changes in the alkanediyl chain linking the two dioxopiperazine rings have resulted in decreased topoisomerase II inhibition. Replacement of the 2,3-butanediyl linking group of ICRF-193 by a 1,2-propanediyl linker gives ICRF-159 (racemic), ICRF-186 (*S*), or ICRF-187 (*R*). All are topoisomerase II inhibitors of similar potency, but about 15–20 times weaker than ICRF-193 [4, 11]. In this case, the absolute configuration of the alkanediyl linker does not affect activity against topoisomerase II significantly. Surprisingly, replacement of the 2,3-butanediyl linker of ICRF-193 by a 1,2-ethanediyl group (ICRF-154) decreases the topoisomerase II inhibition only slightly (5×) [4, 11]. Replacement of the 2,3-butanediyl linker of ICRF-193 with a 2,3-pentanediyl group (ICRF-202) results in only a slight 1.5x decrease in topoisomerase II inhibition, whereas replacement of the 2,3-butanediyl group with a 3,4-hexanediyl group, retaining the meso configuration (ICRF-201), results in a more pronounced (11×) decrease in inhibition [11]. Replacement of the 2,3-butanediyl linker of ICRF-193 with a 1,2-butanediyl linker (ICRF-192) causes a drastic (150×) decrease in topoisomerase II inhibition [11].

Since most modifications of the alkanediyl linker between the dioxopiperazine rings of ICRF-193 decrease but do not reduce inhibition of topoisomerase II drastically, we were surprised by the remarkable inactivity of the (*S,S*)- and (*R,R*)-stereoisomers of ICRF-193. This is also surprising since the absolute configuration at the asymmetric carbon of the alkanediyl linker in ICRF-159, ICRF-186, and ICRF-187 does not affect activity significantly. Although contacts between topoisomerase II and the alkanediyl linker region may be important, other factors may be operative. Substitutions in the alkanediyl linker may either favor or disfavor an active conformation of the two dioxopiperazine rings relative to one another. However, energy differences between various conformations are relatively small when compared to potential enzyme–ligand binding possibilities. It is very possible that hydrophobicity of the linker, influences of configuration on water solubility, and the two methyl functions of the 2,3-butanediyl linker are all important to the enzyme–ligand interaction. Clearly, the (*S,S*)- and (*R,R*)-stereoisomers of ICRF-193 either produce unfavorable conformations and topographies for enzyme interaction or have physical-chemical differences that are incompatible with enzyme binding. For example, the (*R,R*)- and (*S,S*)-enantiomorphs (**1a** and **1b**) were more water soluble and less stable in aqueous solution (6% hydrolyzed in 3 hr, 50% in 21 hr, determined by ^{1}H NMR in D_2O). Tetraacids and tetraamide analogues are also ineffective as topoisomerase II inhibitors, and it is likely that acid-amide hydrolysis products are inactive. However, it should be noted that physical–chemical differences between bis(di-

oxopiperazine)s have not correlated with activity. Racemic ICRF-159 and its *R*-isomer, ICRF-187, differ substantially in solubility but have similar anticancer activities [23] and are approximately equally effective as topoisomerase II inhibitors [11]. Conformationally restricted analogs of the bis(2,6-dioxopiperazine)s have been studied for biological activity [9], but only one such compound (ICRF-197) has been tested for activity against topoisomerase II [11]. Considerations such as these, as well as other physico–chemical properties, may lead to a second generation of conformationally restricted bis(2,6-dioxopiperazine) topoisomerase II inhibitors.

Although cytotoxicity of bis(2,6-dioxopiperazine)s covers a 30,000-fold range and topoisomerase II inhibition a 150-fold range, the two activities are correlated [11]. In general, the strongest inhibitors of topoisomerase II are the most cytotoxic, suggesting that the cytotoxicity is due to topoisomerase II inhibition. A common assumption in cancer chemotherapy is that cytotoxic drugs selectively target rapidly dividing cancer cells. Possibly, cytotoxicity and topoisomerase II inhibition are correlated with efficacy against experimental cancers. ICRF-193 is 1700 times more cytotoxic to mouse L cells than ICRF-196, a racemic mixture of the (*S,S*)- and (*R,R*)-isomers [9]. The inactivity of the (*S,S*)- and (*R,R*)-isomers as topoisomerase II inhibitors is consistent with the idea that such inhibition is the basis for cytotoxicity with this class of drugs.

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