Study of *trans*-Cyclopropylbis(diketopiperazine) and Chelating Agents Related to ICRF 159. Cytotoxicity, Mutagenicity, and Effects on Scheduled and Unscheduled DNA Synthesis

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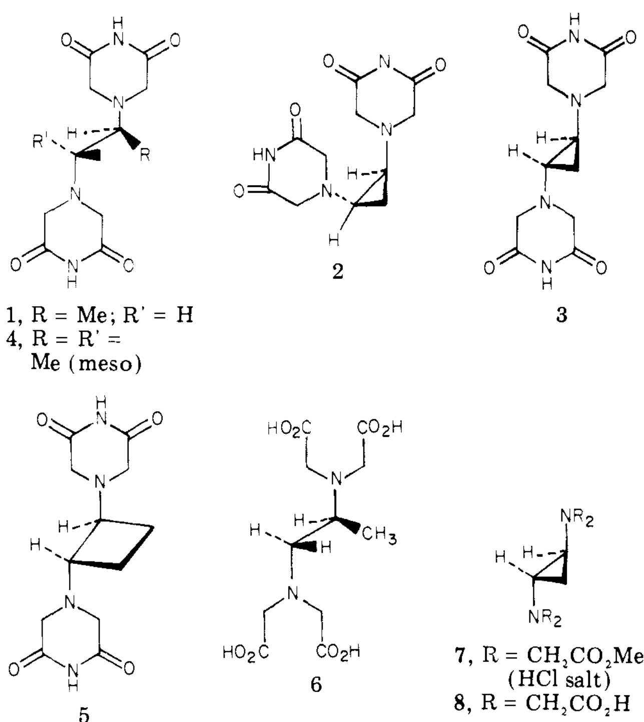
The cytotoxicity, mutagenicity, and DNA damaging potential of *trans*-cyclopropylbis(diketopiperazine) (**3**) and chelating agents related to ICRF 159 (**1**) were examined as a function of concentration and duration of exposure in the Chinese hamster cell line V-79A. At a concentration of 10^{-3} M, **1** and the *trans*-cyclopropanediamine tetraacid **8** and ester **7** proved to be cytotoxic and mutagenic. The *trans*-cyclopropyl analogue **3** of ICRF 159 and acyclic tetraacid **6** were less cytotoxic at all concentrations; analogue **3** exhibited no mutagenic activity at any of the concentrations tested. Compounds **1**, **7**, and **8**, at lethal concentrations, exhibited significantly different mutation frequencies with **7** being sixfold more mutagenic than **8** at the same molar concentration. At 10^{-3} M compound **8** was several times more effective in blocking DNA replication than other analogues but did not induce unscheduled DNA synthesis as did **1**, **3**, and **6**. With the exception of **8**, there was an excellent correlation between mutagenesis and the induction of unscheduled DNA synthesis.

Several bis(diketopiperazines), originally synthesized by Creighton[1,2] as potential intracellular activated chelating agents, exhibit antitumor activity against sarcoma 180 and leukemia L1210 tumors in mice[3] and inhibit [^{3}H]thymidine incorporation into the cellular DNA of mouse embryo fibroblasts.[2,4] One analogue, ICRF 159 (**1**), which has received considerable attention during the past several years,[5-10] is an inhibitor of DNA synthesis[11] and also has been shown to block the cell cycle in G_2.[12-15] Further, **1** inhibits metastasis of Lewis lung carcinoma and prevents alterations in the endothelial lining of blood vessels.[16-20] Preliminary clinical results for anticancer activity of **1** have been reported.[21,22]

It seemed to us that *cis*- and *trans*-cyclopropyl analogues **2** and **3** should represent ideal molecules for assessing stereochemical requirements for biological activity since the molecular weights of the acyclic (**1**) and cyclic (**2** and **3**) compounds differ only by two H atoms. Therefore, the lipophilicity of acyclic and cyclic analogues should not differ markedly and differences in activity should better reflect differences in molecular geometry. Creighton[2] had previously shown that the meso isomer **4**, which is expected to be most stable in the anti conformation, was more potent than **1** in blocking [^{3}H]thymidine incorporation into the DNA of mouse-embryo fibroblasts, while the *dl* isomer of **4** was inactive. The *trans*-cyclobutyl analogue **5**, which is stereochemically related to *dl*-**4** (when the diketopiperazine functions are in the trans conformation), was considerably less potent than either **1** or *meso*-**4** when assessed for its ability to block [^{3}H]thymidine incorporation into mouse-embryo fibroblasts.[2] Furthermore, ethylenediaminetetraacetic acid (EDTA) and ester derivatives of EDTA had been reported to be inactive.[1,2] In many instances biological results obtained with conformationally constrained small-ring analogues cannot easily be interpreted on the basis of the necessity of a certain preferred conformation for a given biological activity.[23-30] Hence, we decided to study analogues with geometry held constant (trans). In this article we compare the biological activity of acyclic bis(diketopiperazine) **1** and tetraacid **6** control analogues with trans cyclic bis(diketopiperazine) **3**, tetraester **7**, and tetraacid **8**. Their cytotoxic and mutagenic activities and their effects on scheduled and unscheduled DNA synthesis in Chinese hamster fibroblast cell cultures (V-79A) are reported.

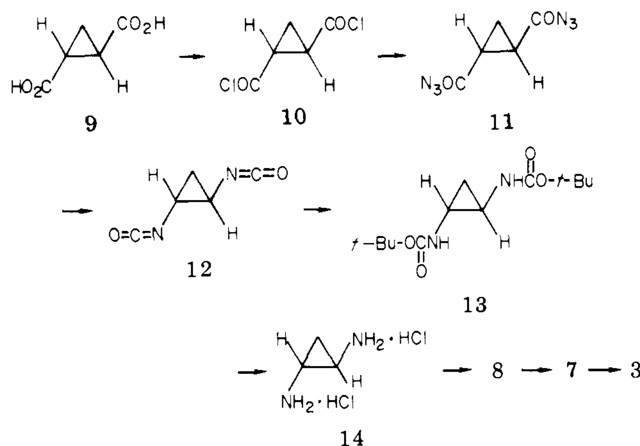


Synthesis. *trans*-1,2-Cyclopropanedicarboxylic acid (**9**), synthesized according to Payne,[31] was converted to the known diacid chloride **10**.[32,33] Although the diamine dihydrochloride **14** had previously been synthesized in low yield from the diethyl ester of **9** via the dihydrazide and bis(benzylcarbamate) intermediates,[34] no physical properties or elemental analyses for **14** were reported. In our laboratories, **14** was most conveniently synthesized from **10** by reaction with NaN_3,[35] Curtius rearrangement (**11** → **12**),[36] and treatment with *t*-BuOH,[37] affording dicarbamate **13** in 54% overall yield. Acid-catalyzed hydrolysis[37] of **13** in EtOH afforded **14** in 88% yield. Reaction of **14** with monochloroacetic acid in aqueous NaOH solution[38] yielded **8** in 94% yield. Tetramethyl ester **7**, isolated as the HCl salt, was synthesized from **8** in 88% yield by stirring overnight in 2,2-dimethoxypropane containing concentrated HCl.[39] Intermediate **7** was converted to bis(diketopiperazine) **3** in poor yield by dissolving **7** in MeOH containing excess NH_3 and NaOMe.[40] Although we were able to synthesize **1** from **6** in good yield according to the method of Creighton,[1] conversion of **8** to **3** under such conditions failed owing to decomposition which took place above 110 °C. The tetramethyl ester **7** was also unstable

Table I. Mutation Frequency Analysis with the Chinese Hamster Cell Line V-79A

Compd	Concn, M	No. of cells per plate	% survival[a]	Total no. of mutants	Mutation frequency per 5×10^5 cells
1	10^{-3}	5×10^5	0.05	8	1600
	10^{-4}	5×10^5	1	12	120
	10^{-4}	1×10^5	1	2	100
	10^{-5}	5×10^5	5	24	48
	10^{-5}	1×10^5	5	4	40
3	10^{-3}	5×10^5	30	6	2
	10^{-3}	1×10^5	30	1	2
	10^{-4}	5×10^5	50	9	2
	10^{-4}	1×10^5	50	2	2
	10^{-5}	5×10^5	70	7	1
	10^{-5}	1×10^5	70	2	2
6	10^{-3}	5×10^5	1	2	50
	10^{-4}	5×10^5	85	12	20
	10^{-4}	1×10^5	85	1	18
	10^{-5}	5×10^5	94	10	11
	10^{-5}	1×10^5	94	2	11
7	10^{-3}	5×10^5	0.02	8	4000
	10^{-3}	1×10^5	0.02	2	5000
	10^{-4}	5×10^5	2	9	45
	10^{-4}	1×10^5	2	2	50
	10^{-5}	5×10^5	90	7	10
	10^{-5}	1×10^5	90	2	12
8	10^{-3}	5×10^5	0.04	3	750
	10^{-4}	5×10^5	0.60	2	34
	10^{-5}	5×10^5	10	5	5
	10^{-5}	1×10^5	10	1	5
Control		5×10^5	100	2	2
		1×10^5	100		
		5×10^5	100	3	3
		1×10^5	100	1	5

[a] Standard deviations for these data are as illustrated in Figure 1.



at elevated temperatures. For example, when 100 mg of the HCl salt of 7 was refluxed in MeOH for 8 h, 60 mg (66%) of $HN(CH_2CO_2Me)_2 \cdot HCl$ [mp 176–177 °C (lit.[41] mp 183 °C). Anal. C, H, N] was isolated.

Biological Results. Cytotoxicity.[42] Figure 1 summarizes the effects of various concentrations of analogues **1**, **3**, **6**, **7**, and **8** on the colony-forming ability (survival) of V-79A following a 24-h exposure to these agents. Neither the parent compounds **1** and **6** nor their cyclopropyl analogues **3**, **7**, and **8** were toxic at concentrations of 10^{-7} M or less. Compounds **1** and **8** were equally toxic, exhibiting a 7- to 20-fold greater toxicity at 10^{-5} M than any of the other compounds. At concentrations greater than 10^{-4} M, tetraester **7** produced the same cytotoxicity as **1** and **8**. The slope of the curve (indicating increasing toxicity with increasing dose) was greater in the case of compounds **6** and **7**. However, since the shoulder of these curves was greater than those of compounds **1** and **8**, toxicity at lower concentrations for these compounds was not as great. Even at the highest concentrations examined (5×10^{-3} M), cyclopropylbis(diketopiperazine) (**3**) was at

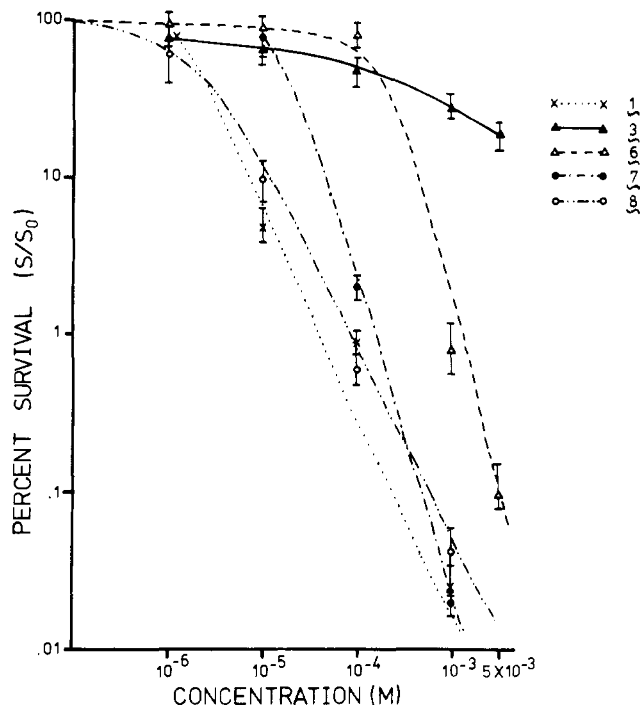


Figure 1. Dose–survival curves for V-79A in the presence of various concentrations of analogues: **1**, ×–×–×; **3**, ▲–▲–▲; **6**, △–△–△; **7**, ●–●–●; **8**, ○–○–○. Standard deviations are indicated for each concentration; average of three independent experiments.

least two orders of magnitude less toxic than any other compound examined.

Mutagenicity.[42] Table I summarizes the frequency of formation of cell variants in culture resistant to a concentration of 1.0 mM ouabain. Each analogue was tested at three concentrations for 24 h for cellular toxicity. Concurrently, cells were treated for 24 h with ICRF 159

Table II. Percent Cells Performing DNA Synthesis 10 h Postapplication of Analogue[a]

Compd	Concn, M[b] 0	10^{-3}	10^{-4}	10^{-5}
1	80.1 ± 1.3	52.2 ± 2.4	57.7 ± 7.1	68.2 ± 4.3
3	83.3 ± 1.5	76.3 ± 1.4	78.3 ± 3.1	82.2 ± 2.6
6	81.7 ± 1.6	61.1 ± 5.1	64.7 ± 6.2	69.2 ± 5.7
8	85.2 ± 2.1	7.3 ± 0.8	75.5 ± 1.2	83.8 ± 2.1

[a] Autoradiograms performed with Ilford-4 emulsion with a 4-day developing time at 4 °C. [^{3}H]Thymidine concentration equals 0.1 µCi/mL (sp act. = 5 Ci/mM) in modified MEM(E) + 10% FBS. Five hundred cells/point examined at 100×. [b] Standard deviations are indicated at each concentration.

or related analogues and subsequently exposed to ouabain in order to determine the number of ouabain-resistant variants induced by the same analogue concentrations used to assess cytoxicity. Compound **3** (Table I) was not mutagenic even at concentrations (10^{-3} M) causing cytotoxicity. While **1** and **8** exhibited similar cytotoxicities, their mutagenic potential varied between two- and eightfold. At similar toxicities **6** and **7** caused a similar mutation frequency, but at 10^{-3} M compound **7** was considerably more cytotoxic and mutagenic.

Scheduled DNA Synthesis.[43] Table II summarizes the effects of analogues **1**, **3**, **6**, and **8** on DNA synthesis. A solution of each analogue, followed by immediate addition of [^{3}H]thymidine, was added to the previously seeded cell cultures. At 0, 10, and 20 h, coverslips were removed and fixed for autoradiographic studies. The population doubling time for these cultures is approximately 12 h. Within 10 h an average of 83% of the cells under nontreated conditions performed DNA synthesis. At 10^{-3} M compound **8** was the most effective blocker of DNA synthesis. Although both **1** and **8** had similar toxicities at 10^{-3} M, **1** was less effective than **8** in blocking [^{3}H]thymidine uptake. Both **1** and **8** were more potent inhibitors of DNA synthesis than **3** and **6** at 10^{-3} M. At 10^{-5} M, **6** was a more effective blocker than **8** and approximately equal to **1** in this assay. At 10^{-3} M, **3** was a weak inhibitor of DNA synthesis.

Unscheduled DNA Synthesis.[43] Table III summarizes the effects of **1**, **3**, **6**, and **8** on the induction of unscheduled DNA synthesis. Dissolved compounds were added to cell cultures 10 h after incubation in media containing 2×10^{-3} M hydroxyurea (this reagent blocks semiconservative DNA replication). [^{3}H]Thymidine was added immediately following addition of the analogues. Coverslips were removed at 0, 3, 5, and 8 h and fixed for autoradiographic analysis. Grains per nuclei increased as a linear function of time. Analogue **1** was the most potent inducer of unscheduled DNA synthesis. The induction of unscheduled DNA synthesis by **1** was linear with time and extrapolated back through the origin suggesting a direct effect not requiring metabolic activation by the cell system. Cyclopropyl analogue **3** was inactive at all concentrations and time periods with the exception of 10^{-3} M following an 8-h incubation. Acyclic tetraacid **6** was inactive for the first 5 h following application. In the 8-h sample, however, **6** proved to be a potent inducer of unscheduled synthesis. This is in agreement with the mutagenic activity of **6**. Compound **8**, like the other three analogues, caused an increase in the number of grains per nuclei as a function of time at any given concentration. However, unlike the other analogues, **8** gave an increase in the number of grains per nuclei as a decreasing function of concentration. Analogue **8** alone induced measurable amounts of unscheduled DNA synthesis at concentrations of 10^{-4} M or less.

Discussion

The observed cytotoxicity for ICRF 159 (**1**) against V-79A fibroblasts in tissue culture agrees with results reported in other systems.[3] The relatively nontoxic nature of *trans*-cyclopropyl analogue **3** also is in accord with the reported decreased activity for the *trans*-cyclobutyldiketopiperazine **5** in the sarcoma 180, leukemia L1210, and [^{3}H]thymidine assays.[2] The decreased activity observed for analogues **3** and **5** may be a reflection of their structural relationship to *dl*-**4** which is reported to be inactive.[2] Since *meso*-**4** is very potent,[2] *cis*-diketopiperazine **2** and the cis isomer of **5** are of particular interest and will be the subject of a future report. Although *meso*-**4** is expected to be in a preferred trans conformation in solution, it may well be that this analogue interacts with an enzyme in the conformation of a cis-1,2-disubstituted cyclopropane or cyclobutane.

For cytotoxicity, the order in increasing potency ($3 < 6 < 7 < 8 \sim 1$) does not follow the exact order of increasing mutagenicity ($3 < 6 < 8 < 1 \leq 7$). However, those compounds which were among the most mutagenic were also among the most cytotoxic. Nonetheless, owing to differences in chemical properties between imides (**1** and

Table III. Comparative Influence of Analogues on Unscheduled DNA Synthesis[a]

Compd	Time, h[b]	Concn, M[c] 0	10^{-3}	10^{-4}	10^{-5}
1	0	0.2 ± 0.03	0.4 ± 0.08	0.2 ± 0.07	0.4 ± 0.12
3		0.5 ± 0.08	0.2 ± 0.04	0.2 ± 0.08	0.6 ± 0.09
6		0.3 ± 0.05	0.4 ± 0.11	0.2 ± 0.05	0.3 ± 0.08
8			0.3 ± 0.06	0.3 ± 0.07	0.4 ± 0.09
1	3	0.4 ± 0.1	4.1 ± 0.3	2.8 ± 0.1	2.2 ± 0.3
3		0.5 ± 0.1	0.8 ± 0.2	0.6 ± 0.3	0.8 ± 0.1
6		0.4 ± 0.2	1.2 ± 0.2	1.0 ± 0.2	0.9 ± 0.2
8		0.6 ± 0.2	0.5 ± 0.1	1.0 ± 0.2	2.2 ± 0.1
1	5	0.9 ± 0.3	17.2 ± 0.6	5.4 ± 0.6	2.6 ± 0.2
3		1.0 ± 0.2	1.5 ± 0.3	2.0 ± 0.3	1.5 ± 0.2
6		0.8 ± 0.2	2.6 ± 0.1	2.5 ± 0.2	1.5 ± 0.1
8		1.1 ± 0.3	0.9 ± 0.2	2.1 ± 0.2	4.1 ± 0.3
1	8	1.6 ± 0.2	28.9 ± 2.0	9.5 ± 2.0	4.6 ± 0.3
3		1.1 ± 0.3	3.4 ± 1.1	2.0 ± 0.9	1.9 ± 0.4
6		2.1 ± 0.4	11.8 ± 0.8	6.0 ± 1.1	3.1 ± 0.5
8		1.5 ± 0.5	1.3 ± 0.3	4.2 ± 0.7	5.6 ± 0.3

[a] Analogue influence on [^{3}H]thymidine uptake was assessed after prior addition of 2×10^{-3} M hydroxyurea. [b] Uptake of [^{3}H]thymidine was assessed at this time after addition of analogues and [^{3}H]thymidine to the cell cultures. [c] Each point represents the average number of grains per nuclei for a total of 400 cells. Standard deviation is indicated at each concentration.

3) and tetraacids (6 and 8), we would anticipate the tetraacids, which are strong chelating agents, to exert their effects by different modes of action than diketopiperazine analogues. For bis(diketopiperazines) the rigid trans geometry for 3 renders the compound less toxic and mutagenic than its acyclic analogue 1. For the tetraacids, the rigid trans geometry of 8 renders the compound more toxic at all concentrations and generally more mutagenic than its acyclic analogue 6. At the lowest concentration examined, 10^{-5} M, the apparent equivalent mutagenicity between these two compounds may be real or reflect the difficulties encountered when examining the mutagenic potential of weak mutagens. If sequestering cations in the medium were responsible for the cytotoxic effect, both 6 and 8 would be expected to be equally active; if asymmetric "error free postreplication gap filling" enzymes[44] are involved in the chelating mechanism, one might anticipate a difference in activity between 6 and 8, and such differences in mutagenicity were observed. The fact that tetraester 7 is more mutagenic than tetraacid 8 may be a reflection of enhanced cellular uptake of this nonzwitterionic compound.

At 10^{-3} M, cyclopropyl tetraacid 8 was the most effective blocker of scheduled DNA synthesis, one of the most cytotoxic and mutagenic agents studied, but a weak inducer of unscheduled DNA synthesis. Since DNA damage is a prerequisite for mutagenesis,[44] these observations appear to be contradictory. In fact, recent studies[45] indicate that several agents including 7-bromomethylbenz[*a*]anthracene in Chinese hamster V-79A cultures afford results similar to those observed in these studies. At higher concentrations of this hydrocarbon (exhibiting the greatest cytotoxic and mutagenic potential) the lowest level of unscheduled DNA synthesis was observed.[45] Most agents which inhibit scheduled DNA synthesis also inhibit unscheduled DNA synthesis.[44] One possible explanation for the differential action of 8 is that at higher concentrations 8 inhibits asymmetric enzymes (perhaps via metal chelation) involved in scheduled as well as unscheduled DNA synthesis, thus permitting DNA damage, caused by the compound, to enter DNA replication with the subsequent activation of an "error prone" postreplication repair[44] process. Recent studies strongly indicate that such an "error prone" process can be induced in V-79A cell cultures by agents known to inhibit DNA replication.[46,47]

All other analogues (1, 3, and 6) examined showed a decreased ability to inhibit scheduled DNA synthesis and induced unscheduled DNA synthesis as a direct function of dose. Again, the considerably decreased effect of cyclopropylbis(diketopiperazine) (3), as compared to ICRF 159 (1), is likely a reflection of its unfavorable rigid trans geometry. Unlike cyclopropyl tetraacid 8, acyclic tetraacid 6 exhibited only marginal effects on scheduled DNA synthesis; this observation is consistent with the lack of cytotoxicity reported for acyclic tetraacetic acids.[1,2] Interestingly, acyclic tetraacid 6 is more like acyclic bis(diketopiperazine) 1 than like cyclic tetraacid 8 with respect to its effects on unscheduled and scheduled DNA synthesis particularly at a concentration of 10^{-3} M. Other than invoking the clear differences in chemical properties between cyclic and acyclic systems and between acids and imides, which would suggest potential differences in mechanisms of action, further explanation of these results would be premature.

Experimental Section

Chemistry. For obtaining physical data on our compounds the following instruments were employed: melting points, calibrated Thomas-Hoover apparatus; IR spectra, Perkin-Elmer 257 spectrophotometer; NMR spectra, Varian A-60A spectrometer; GLPC, Hewlett-Packard 402 biomedical gas chromatograph. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. 37921.

***trans*-1,2-Cyclopropanedicarboxylic acid (9)** was prepared according to the method of Payne[31] affording crystals, mp 176–177 °C (lit.[48] mp 177–177.5 °C).

***trans*-1,2-Cyclopropanedicarbonyl Dichloride (10).** A mixture of 47.01 g (0.36 mol) of *trans*-diacid 9, $SOCl_2$ (160 g, 1.34 mol), and benzene (300 mL) was heated (60–70 °C) for 24 h with stirring. Excess $SOCl_2$ was removed under reduced pressure and the residue was distilled affording 51.9 g (86.2%) of 10, bp 42–44 °C (2 mm) [lit.[32] 67–68 °C (5 mm)].

Di-*tert*-butyl *trans*-1,2-Cyclopropanediylbis(carbamate) (13). Compound 10 (51.9 g, 0.31 mol) in Me_2CO (80 mL) was added dropwise to an aqueous solution of NaN_3 (67 g, 1.03 mol) in H_2O (160 mL) with stirring and cooling in an ice bath.[35] The reaction mixture was stirred for an additional 2 h and poured into ice-H_2O (300 mL). The product was extracted with Et_2O and the organic layer was washed with H_2O and dried ($MgSO_4$). The Et_2O was removed under reduced pressure affording 59 g of liquid showing IR (neat) 1705 and 2160 cm^{-1} for azide 11. Azide 11 was heated in toluene (1 L) on a steam bath until evolution of N_2 ceased.[36] The toluene was removed under reduced pressure affording a yellow liquid exhibiting IR (neat) 2300 cm^{-1} for isocyanate 12. Isocyanate 12 was refluxed in *t*-BuOH (350 mL) for 2 h.[37] After removal of the solvent under reduced pressure the resulting solid residue was purified by chromatography on silica gel 60 (70–230 mesh) using hexane–$CHCl_3$–MeOH (10:9:1) as the eluting solvent. The solvent eluate containing 13 (TLC) was concentrated under reduced pressure and the residue was recrystallized from benzene affording 46.1 g (54.3% based on starting 10) of carbamate 13: mp 131–132 °C; IR (KBr) 3340 (NH), 1680 (carbamate C=O), 1390, 1370 cm^{-1} (*t*-Bu); NMR ($CDCl_3$) δ 1.00 (t,[50] 2, CH_2), 1.43 (s, 18, *t*-Bu), 2.43–2.67 (m, 2, CHN), 5.02 (s, 2, NH). Anal. ($C_{13}H_{24}N_2O_4$) C, H, N.

***trans*-1,2-Cyclopropanediamine Dihydrochloride (14).** A solution of 25.7 g (0.094 mol) of carbamate 13 in 95% EtOH (500 mL) containing 30 mL of concentrated HCl was refluxed with stirring for 5 h.[37] Removal of the solvent under reduced pressure afforded 11.72 g (88%) of diamine dihydrochloride 14 as white crystals. Recrystallization from MeOH–Et_2O afforded 14: mp 210 °C dec; IR (KBr) 3200–2500 cm^{-1} (br, $-NH_3^+$; NMR (D_2O[51]) δ 1.53 (t,[50] 2, CH_2), 3.18 (t,[50] 2, CHN). Anal. ($C_3H_8N_2{\cdot}2HCl$) C, H, N.

***trans*-2,2′,2″,2‴-(1,2-Cyclopropanediyldinitrilo)tetrakis(acetic acid) (8).** To a cooled solution (10 °C) of monochloroacetic acid (37.8 g, 0.41 mol) in H_2O (20 mL) was added slowly, and with stirring, a cold solution of NaOH (32 g, 0.8 mol) in H_2O (66 mL) through use of a dropping funnel. The temperature was not allowed to exceed 20 °C. To this mixture, 10 g (0.07 mol) of 14 was added. The mixture was covered with argon gas and allowed to stand for 5 days at 20 °C.[38] The reaction mixture was acidified (pH 1.2) with 9 N H_2SO_4 and kept at 4 °C overnight. The yellow-brown crystals were filtered and washed with cold H_2O followed by MeOH and Me_2CO. After drying, 19.84 g (94.5%) of crude product 8 was obtained. The crude material was dissolved in 4% aqueous NaOH solution (160 mL), decolorized with charcoal, and reacidified (pH 1.2). White crystals were obtained on standing at 4 °C overnight. Recrystallization from EtOH–H_2O (1:1) afforded 8: mp 176–178 °C dec; IR (KBr) 1700 cm^{-1} (CO_2H); NMR (D_2O–NaOH[51]) δ 1.43 (t,[50] 2, CH_2), 3.38 (t,[50] 2, CHN), 3.73 (s, 8, NCH_2CO). Anal. ($C_{11}H_{16}N_2O_8$) C, H, N.

Tetramethyl-*trans*-2,2′,2″,2‴-(1,2-cyclopropanediyldinitrilo)tetrakis(acetic acid) Dihydrochloride (7). A mixture of 9.43 g (0.033 mol) of tetraacetic acid 8 in 2,2-dimethoxypropane (450 mL) containing 30 mL of concentrated HCl was stirred at room temperature for 18 h.[39] The dark-brown reaction mixture was concentrated under reduced pressure and the residue was dissolved in 100 mL of MeOH. Addition of 300 mL of Et_2O resulted in crystallization of 7. Recrystallization from MeOH–Et_2O (room temperature) afforded 11.9 g (88.8%) of pure dihydrochloride salt 7: mp 156–157 °C; IR (KBr) 2930–2400 (br, R_3NH^+), 1750, 1220 cm^{-1} (ester); NMR (D_2O[51]) δ 1.37 (t,[50] 2, CH_2), 3.35 (t,[50] 2, CHN), 3.80 (s, 12, OCH_3), 4.05 (s, 8, NCH_2CO). Anal. ($C_{15}H_{24}N_2O_8{\cdot}2HCl$) C, H, N.

The free base of 7 was obtained by dissolving the dihydrochloride salt in 10% aqueous Na_2CO_3 solution and extracting with Et_2O. The dried Et_2O solution ($MgSO_4$) was concentrated under reduced pressure and the liquid residue exhibited by GLC on 10% silicone gum rubber one major peak (97.8%) with a small shoulder (2.2%): IR (neat) 1730 (ester, C=O), 1430 (CH_3), 1170 cm^{-1} (ester COC); NMR ($CDCl_3$) δ 0.77 (t,[51] 2, CH_2), 2.65 (t,[51] 2, CHN), 3.60 (s, 8, NCH_2CO), 3.70 (s, 12, OCH_3).

***trans*-4,4′-(1,2-Cyclopropanediyl)bis(2,6-piperazinedione) (3).** To a solution of the free base 7 (5.33 g, 0.015 mol) in MeOH (400 mL) was added 20 mL of a solution containing 7.1 g (0.4 mol) of NH_3 in 100 mL of MeOH and NaOMe (1.62 g, 0.03 mol).[40,49] The solution was stirred at room temperature for 3 days; each day the NH_3–MeOH solution (20 mL) and NaOMe (0.81 g, 0.015 mol) were added. The solvent was removed under reduced pressure and the residue was dissolved in H_2O (200 mL). The aqueous solution was cooled in an ice bath and adjusted to pH 7–8 with 2 N HCl. After standing at 4 °C overnight the crystals were filtered and washed with successive portions of cold H_2O, MeOH, and Me_2CO. The resulting crystals were washed with 10% aqueous $NaHCO_3$ solution. The crystals were dissolved in 7% Na_2CO_3 solution (200 mL) and acidification (pH 3) followed by standing at 4 °C overnight afforded 1.06 g (27%) of crystalline white diimide **3** after filtration and washing with successive portions of cold H_2O, MeOH, and Me_2CO. Recrystallization from dioxane afforded pure **3**: mp >300 °C dec; mass spectrum *m/e* 266; IR (KBr) 3200, 3100 (NH), 1730, 1690 cm^{-1} (imide); NMR (D_2O–NaOH[51]) δ 0.88 (t,[50] 2, CH_2), 2.00 (t,[50] 2, CHN), 3.33 (s, 8, NCH_2CO). Anal. ($C_{11}H_{14}N_4O_4$) C, H, N.

Biology. Stock solutions of analogues were prepared in PBS (phosphate buffered saline solution) affording concentrations of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} M. For insoluble analogues **1**, **3**, **6**, and **8** the pH was adjusted to approximately 8 for the 10^{-2} M stock solution; 1-mL aliquots of these solutions were used to prepare subsequent dilute solutions. A 1-mL aliquot of each stock solution was added to the petri dish containing 9 mL of media to afford the desired concentration of drug (10^{-3}–10^{-6} M).

Cell Culture. Cultures were grown in 32-oz prescription bottles containing 25 mL of tissue culture medium Eagle's minimal essential medium supplemented with 2 mmol of glutamine (Flow Lab.), 0.11% $NaHCO_3$, 5% fetal calf serum (Flow Lab.), nonessential amino acids (Microbiological Associates), 50 μg/mL of gentamiacin sulfate (Shering Corp.), and 1 mmol of sodium pyruvate, pH 6.9–7.1. Confluent cells were washed twice in Hank's basic salt solution and detached with Hank's basic salt solution minus Mg^{2+} and Ca^{2+} containing 30 μg/mL of crystalline trypsin (Worthington Biochemical) and 0.01% methylcellulose. Cells were subsequently dispersed with a large-bore pipet, diluted twofold, and plated.[23]

Cytotoxicity Studies.[23,42] Cells were seeded at densities of 250, 500, and 1000 cells per 100-mm diameter petri dish and permitted to attach for 2 h at which time the medium was changed to Eagle's minimal essential medium (modified as above) plus 15% fetal calf serum containing a concentration of either 10^{-3}, 10^{-4}, 10^{-5}, or 10^{-6} M drug or no drug. At this time a set of ten control plates were fixed and stained as described below and examined for multicellular foci. Owing to the method described above, in which cells in division are washed off prior to detachment of the cell sheet, no occurrence of multicellular foci was expected or observed. After 24 h, this medium was poured off and the cells were rinsed twice with 10 mL of Hank's basic salt solution. New medium, not containing drugs, was added and the cells were incubated at 37 °C in a 5% CO_2 humidified incubator for 7 days. Cells were fixed and stained in Harris hematoxylin with an eosin counterstain and the number of colonies per plate counted. Each point represents the average of eight plates.

Mutagenesis.[42] Cells were seeded into 100 mm diameter petri dishes at a concentration of 5 or 1 × 10^5 cells per plate in Eagle's minimal essential medium (modified as above) plus 5% fetal calf serum and permitted to attach for 2 h. At this time, cells from ten control plates were detached and the cell number was determined. On all other plates, test compounds were added to obtain a final concentration of 10^{-3}, 10^{-4}, or 10^{-5} M drug or no drug and incubated at 37 °C in 5% CO_2. After 24 h, the medium was poured off and the cells were washed twice with 10 mL of PBS and a new minimal essential medium (modified as above) plus 10% fetal calf serum containing 10^{-3} M ouabain was added. After 12 days, cells were fixed in Carnoy's and stained in Harris hematoxylin with an eosin-counter stain and the number of surviving colonies per plate was counted. The result for each drug, at each concentration, represents the average of 20 plates.

Radioautography.[23,43] Cells were seeded at a density of 10^4 cells/cm^2 in 100-mm diameter petri dishes, in which 22 × 11 mm coverslips had been placed. Cells were permitted to attach for 24 h at which time the medium was changed to Eagle's minimal essential medium (modified as above) plus 15% dialyzed fetal calf serum containing 2 × 10^{-3} M hydroxyurea (unscheduled DNA synthesis studies). After 6 h, the hydroxyurea containing medium was poured off and new medium (as above) containing hydroxyurea and drugs at 10^{-3}, 10^{-4}, 10^{-5} or 0 M concentration was added in conjunction with [^{3}H]thymidine at a concentration of 2 μCi/mL (sp act. = 15 Ci/mmol) so as to measure unscheduled DNA synthesis. Treated and control samples were incubated for various times from 0 to 24 h. At the end of the incorporation period, coverslips were washed with a nonradioactive balanced salt solution, fixed in Carnoy's solution for 10 min, and prepared for radioautography by a standard procedure. The coverslips were dipped into twofold diluted Ilford No. 4 emulsion and allowed to expose for 6–7 days. Slides were developed in D-19, fixed, and stained in Harris hematoxylin. Approximately 400 cells were observed at a magnification of 200-fold and the number of grains for each cell was counted.

The same technique was used in the study of scheduled DNA synthesis with the exception that the culture media did not contain hydroxyurea.

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(50) Deceptively simple triplet.
(51) D_2O + 1% (w/w) sodium 2,2-dimethyl-2-silapentane-5-sulfonate; 98.8% D.

Nonenolizable Podophyllotoxin Derivatives

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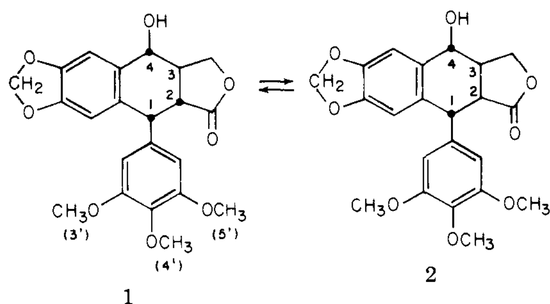
To block epimerization and the resulting biological deactivation of podophyllotoxin compounds, the lactone carbonyl group has been changed to methylene. Syntheses of several of these delactonized derivatives are described, all with modifications in the original lactone ring and some without the 4-hydroxyl group. Several biological assays show that most of the nonenolizable derivatives retain activity.

Podophyllotoxin (1) and several of its analogues and derivatives are cytostatic spindle poisons;[1] they have also received considerable attention as antitumor agents,[2-6] some at the clinical level. Most of these compounds contain a trans-fused highly strained γ-lactone system,[3]



a feature that correlates with the smooth isomerization of podophyllotoxin (1) to its thermodynamically stable cis epimer, picropodophyllin (**2**).[7] The biological activity of picropodophyllin as well as of the other cis analogues is either much lower than that of the trans isomers or is lacking altogether.[4,8,9] Since the epimerization conditions are very mild, the possibility has been considered that, when exposed to podophyllotoxin, the cell makes use of this process for detoxication. A search of the literature has uncovered scattered reports that, in fact, are consistent with the idea of detoxication by epimerization.[9]

From the chemotherapeutic point of view, the epimerization is undesirable, since limiting the physiological lifetime of the compounds would set an upper limit to their biological effectiveness. To get rid of this built-in disadvantage, we initiated a program of synthesizing derivatives in which epimerization is precluded, thus allowing the maximum effect and the full potential of the agents to be realized. Replacing the hydrogen at the 2 position with a suitable group would provide the kind of compounds we were after, and, with this in mind, we have tried to insert a fluorine at this position but, so far at least, have not succeeded.[10] Substituting with a carboxyl or carbomethoxyl group was possible but unfortunately was accompanied by inversion, so that the derivatives were obtained in the unfavorable cis-fused picropodophyllin (**2**) configuration instead of in the trans-fused podophyllotoxin (1) form.[11] A quite different way of blocking epimerization would be to eliminate the lactone group altogether, since without an enolization mechanism, the cell could not bring about the mild inversion. Accordingly, we have now prepared a series of "delactonized" derivatives **3**, in which the original carbonyl group has been changed to methylene,