Cyclopentenylcytosine. A Carbocyclic Nucleoside with Antitumor and Antiviral Properties†

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Cyclopentenylcytosine (CPE-C, **2**), a pyrimidine analogue of the fermentation derived carbocyclic nucleoside neplanocin A, has been synthesized from the optically active cyclopentenylamine **3b** by two synthetic routes. CPE-C demonstrates signifiant antitumor activity against both the sensitive and ara-C resistant lines of L1210 leukemia in vivo. Multiple long term survivors are produced in both tumor models. The compound also gives 100% growth inhibition of the solid human A549 lung and MX-1 mammary tumor xenografts grown in athymic mice. Good activity is also observed against a third human tumor xenograft model, metastatic LOX melanoma. CPE-C has significant activity against both DNA and RNA viruses in vitro. Potent activity is observed against HSV-1 (TK+ and TK−), HSV-2, vaccinia, cytomegalovirus, and varicella-zoster virus. Good activity is also found against a strain of influenza virus (Hong Kong flu), vesicular stomatitis virus, Japanese encephalitis virus, and Punta Toro virus.

Since the report on the synthesis of carbocyclic adenosine[1,2] [(±)-aristeromycin] and its subsequent isolation from natural sources [(−)-aristeromycin],[3-5] numerous carbocyclic nucleoside analogues have been synthesized and studied as potential antitumor and antiviral agents.[6a] More recently, the fermentation product neplanocin A (an unsaturated analogue of aristeromycin) has generated considerable attention both synthetically and biologically because of the effect of the double bond on compound activity and potency.[6a,7] Carbodine (1), the cytosine analogue of aristeromycin, is a compound with established biological activity.[6b] These circumstances prompted us and others to prepare the corresponding unsaturated cytidine analogue **2** (cyclopentenylcytosine, CPE-C) in order to evaluate the biological effect of inserting a double bond into the "carbocyclic sugar" portion of the carbodine molecule.[8-12]

CPE-C was independently synthesized in our laboratory and that of Ohno's in Japan.[8,9] After the publication of these preliminary communications, three recent articles have appeared in which the antitumor activity and the mechanism of action of this compound have been described in more detail.[10-12]

In these studies, the in vitro cytotoxicity of CPE-C was confirmed, and subsequent in vivo experiments suggested that the compound had definite potential as an antitumor agent.[12]

The investigations on the mechanism of action of CPE-C revealed that the cytotoxic effect of the drug was related to the near total abrogation of CTP pools produced in treated cells.[10-12] Interestingly, this effect showed some tissue-specificity toward the tumor, as ascertained by comparing reduction of CTP levels in both normal and tumor tissue of L1210 bearing mice.[12] Taken together, these results suggested that the drug, or a metabolite of it, was inhibiting the conversion of UTP to CTP in the reaction catalyzed by CTP synthetase, one of the rate-limiting reactions in the de novo pyrimidine biosynthetic pathway.[10,11] The reported isolation of the triphosphate metabolite of CPE-C from L1210 cell extracts confirmed that this metabolite was indeed responsible for the inhibition of CTP-synthetase.[13,14] In addition to the

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Scheme I

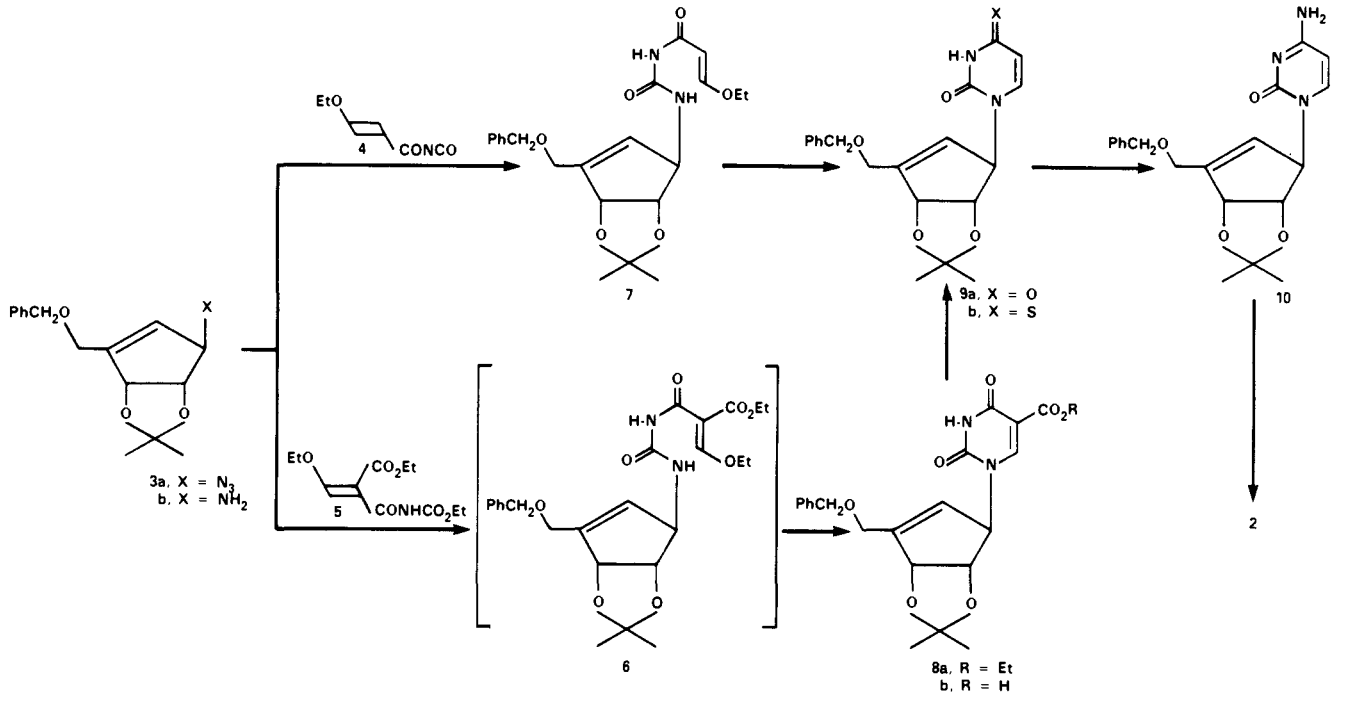
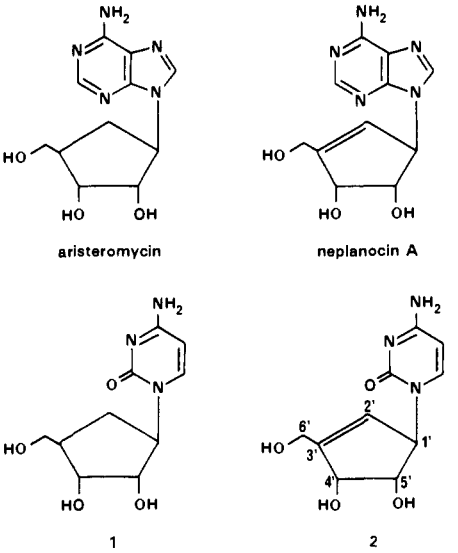


Table I. Effect of Treatment Schedule on the Activity of CPE-C against L1210 Leukemia[a]

CPE-C treatment schedule	optimum dose, mg/kg per inj	ILS,[b] %	LCK[c]
daily, days 1–9	1	124	3.0
every 4th day, days 1, 5, 9	4	91	0.1
every 3 h × 8, days 1, 5, 9	0.5	116	1.5
none[d]		0	−3.0

[a] Intraperitoneal (ip) implantation of 10^5 L1210 cells on day 0 in CD_2F_1 mice. Ip treatment. [b] Median percent increase in life span of tumored, treated mice relative to tumored, untreated controls. Control median survival time was 8.9 days. Survival of saline-treated, tumored mice was 9.0 days. [c] Approximate log cell kill at the end of treatment (ref 28). [d] Saline treatment, all schedules.

aforementioned reduction of CTP levels, CPE-C elicited a very rapid and complete morphologic response in differentiating the HL-60 promyelocytic cell line with the concomitant reduction of *c-myc* RNA levels preceding the appearance of differentiated cells.[11] The key metabolite responsible for all of these activities appears to be the corresponding triphosphate of CPE-C (CPE-CTP). However, in the course of its metabolic conversion to the triphosphate level, CPE-C also inhibited competitively the phosphorylation of uridine, which could contribute in a certain measure to its overall activity.[9,13] It is also important to point out that CPE-C shares a common mechanism of action with carbodine (1) in inhibiting CTP synthesis.[11,15,16] Carbodine has a cyclopentane ring without a double bond, and the observed differences in potency between 1 and **2** suggest that, as with neplanocin A, the unsaturation is a significant factor for increased potency. In reducing CTP levels and inducing differentiation in HL-60 cells, CPE-C was about 100 times more potent than carbodine.[11] A similar potency difference was observed in in vivo antitumor experiments.[12] In this paper we present in detail a convenient synthesis of CPE-C along with antitumor and additional antiviral activity[17,18] for this compound.



Chemistry

Enantiomeric cyclopentenylcytosine (CPE-C, **2**) was prepared as shown in Scheme I from the optically pure cyclopentenylamine **3b** obtained in situ by the catalytic reduction of its azide precursor **3a**. The syntheses of **3a** and **3b** were reported earlier by us in connection with a total synthesis of neplanocin A.[19] With **3b** as the starting material, two well-known routes to pyrimidine nucleosides were explored.[20a-c] In the first approach, the carbocyclic

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Table II. CPE-C Activity against Mouse Tumor Models

		activity[a]					
		experiment 1			experiment 2		
tumor	tumor implant site[b]	OD[c]	%ILS[d]	LCK[d]	OD	%ILS	LCK
L1210	ip	1.0	212 (4)[e]	>6.0	1.0	201 (2)	>5.8
	ip[f]	2.25	125	1.9	2.25	57	−2.2
	sc	1.5	109		1.0	83 (1)	
	ic	1.5	45		1.0	42	
P388	ip	1.0	88	0.9	1.5	69	0.1
P388/ara-C[g]	ip	1.5	165 (3)	>6.5	1.5	133	3.8
M5076	ip	0.5	5	−2.0			

[a] IP, QD1–9 treatment schedule (QD1–13 for M5076). [b] Ip, intraperitoneal; sc, subcutaneous; ic, intracerebral. [c] Optimum dose (mg/kg per injection). [d] See abbreviations in Table I. [e] Number in parentheses equals number of 30-day survivors out of six mice. [f] Oral drug administration. [g] Arabinosylcytosine resistant P388 leukemia.

amine was condensed with 3-ethoxy-2-[[(ethoxycarbonyl)amino]carbonyl]-2-propenoate (Holy's reagent, **5**)[20a] to give intermediate **6**, which without isolation underwent cyclization of the 5-substituted uracil derivative **8a**. Conversion of **8a** to the unsubstituted uracil derivative **9a** was initially achieved by a copper-catalyzed decarboxylation in refluxing quinoline performed on the free acid **8b**. A modification of this decarboxylation using 1,10-phenanthroline and cuprous oxide[21] produced complete conversion to **9a** after only 15 min of reflux. After workup and column chromatography, this procedure afforded a 71% yield of the protected uracil **9a**. Thiation of **9a** to give **9b** was accomplished with Lawesson's reagent,[22] and treatment of the resulting mercaptouracil intermediate with methanolic ammonia afforded the protected CPE-C derivative (**10**) in one step. The preparation of a methylthio intermediate prior to ammonolysis, as reported previously,[9] was found not to be required. Finally, removal of the protective groups with BCl_3 accomplished the final step to give CPE-C (**2**) as a white crystalline solid. In an effort to reduce the number of steps, a second and more direct approach was attempted. The cyclopentenyl amine **3b** was reacted with 3-ethoxy-2-propenoylisocyanate (**4**) to give the stable intermediate **7**. This compound underwent a smooth cyclization to give **9a**, thereby eliminating the need to perform the additional hydrolysis and decarboxylation of the previous approach. The rest of the steps to CPE-C were identical with those already discussed in the first approach.

Antitumor Activity. Earlier, limited in vivo testing against L1210 leukemia indicated that the saturated carbocyclic analogue, carbodine (**1**), and its corresponding "ara" analogue were active, giving increase in life span (ILS) values of 82% and 104%, respectively.[16,23] The 2′- and 3′-deoxy analogues were, however, without activity or toxicity.[24] The initial CPE-C (**2**) in vivo tests against the same tumor model (L1210) showed similar levels of activity, but potencies about 100 times greater than carbodine.[8b,12] Experiments using a strain of L1210 leukemia resistant to arabinosylcytosine (ara-C) indicated that this tumor possessed collateral sensitivity to CPE-C.[12] These results prompted a detailed study of the antitumor activity of CPE-C in a number of in vivo model tumor systems.

Mouse Tumor Models. Since nucleoside antitumor drugs sometimes have a marked treatment schedule dependency, an initial set of experiments was carried out to determine the importance of treatment schedule for CPE-C. Using intraperitoneally implanted L1210 leukemia, CPE-C was active when given on a chronic (QD 1-9), intermittent (Q4D, D 1,5,6), or on an "around-the-clock" (Q3H × 8, D 1,5,9) schedule (Table I). Although the intermittent schedule appeared to be slightly less effective than the other two evaluated, no significant schedule dependency was noted. Most of the subsequent tests were conducted with use of the chronic treatment schedule.

Table III. CPE-C Activity against Human Tumor Xenografts in Athymic Mice[a]

		% tumor inhibition[c] (or % ILS)			
	tumor implant site[b]	experiment 1		experiment 2	
tumor		OD	activity	OD	activity
A549 lung	src	1.5	−59[d]	2.0	92[d]
MX-1 mammary	src	1.5	100	2.25	95
LOX melanoma	ip	1.0	(119)	2.25	(80)

[a] See abbreviations tables I and II. Ip treatment QD1–9. [b] Src, subrenal capsule. [c] Based on relative changes in mean tumor weights of treated and control tumors between day 0 and 11. Negative value denotes tumor regression and was calculated by dividing the change in mean treated tumor weight by the initial mean treated tumor weight and multiplying by 100. [d] This activity associated with weight loss and one or two deaths in each group of six mice.

Table II summarizes the antitumor activity of CPE-C against a standard panel of mouse tumor models used by the National Cancer Institute (NCI). The compound is both potent and highly active in vivo against L1210 leukemia. Multiple 30-day survivors are obtained at an intraperitoneal dose of 1.0 mg/kg on the QD 1–9 treatment schedule. When administered orally, the activity is somewhat lower, but still significant. Reproducible activity of ca. 40% ILS is obtained against intracerebral L1210, but the level is so low that the effect might be attributed to activity against systemic tumor cells that have metastasized from the brain. CPE-C was inactive against the M5076 sarcoma and showed only modest activity against P388 leukemia. However, a P388 leukemia line resistant to ara-C (P388/ara-C) was collaterally sensitive to CPE-C with multiple long term survivors produced in one experiment.

It may be noted that there is a spread of %ILS values showing the effect of CPE-C against intraperitoneal L1210 leukemia using the chronic treatment schedule (QD 1–9). Values shown range from 124% (Table I) to 212% and 201% (Table II) and 176% (Table IV). These results are from four separate experiments conducted by three different testing laboratories over a 16-month period. While the test described in Table I is the only experiment shown that did not produce at least one long-term survivor, it is reported as such, since all the schedule dependency data

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Table IV. Antitumor Activity Comparison between CPE-C and 3-Deazauridine[a]

tumor	CPE-C dose, mg/kg	CPE-C %ILS	3-deazauridine dose, mg/kg	3-deazauridine %ILS
MX-1	2.25	100[b]	300	64[b]
mammary	1.5	94[b]	200	11[b]
xenograft				
LOX	2.25	80	300	4
melanoma	1.5	58	200	5
xenograft				
L1210	2.25	146	100	80
leukemia[c]	1.5	171 (2/9)[d]	50	61
	1.0	116 (1/10)	25	72
L1210/ara-C	2.25	>650 (6/10)	50	166
leukemia[c]	1.5	>650 (5/9)	25	362 (3/9)
	1.0	>650 (6/10)	12.5	>650 (9/10)

[a] QD1–9 treatment schedule. MX-1 subrenal capsule tumor implant site, all others intraperitoneal. Compounds tested in the same experiment. [b] Percent tumor weight inhibition for this solid tumor based on changes in tumor weights on day 0 and day 11. [c] Experiment terminated on day 60 rather than the normal day 30. When >50% of the tumored animals were alive on day 60, the median survival time was not reached. [d] Numbers in parentheses are number of 60-day survivors per test group.

were obtained in the same experiment for direct comparison purposes. The other three values (shown in Table II and IV), are within a range that can be expected for a very active compound with a steep dose–response curve.

Human Tumor Xenograft Models. CPE-C has good activity against all three of the human tumor xenograft models used in the NCI tumor panel (Table III). Two human solid tumors (A549 lung and MX-1 mammary) grown under the renal capsule of athymic ("nude") mice were sensitive to the compound, and tumor regressions were noted in one of the lung tumor experiments, albeit with indications of toxicity. CPE-C was active against the human metastatic melanoma line, LOX, giving ca. 100% increase in life span against this intraperitoneally implanted model.

Comparison with 3-Deazauridine. The biological profile of CPE-C has several similarities to that of 3-deazauridine (3DU). Both compounds are powerful inhibitors of CTP synthesis.[10,25] Also, tumors resistant to ara-C are collaterally sensitivie to 3DU[26] and CPE-C (Table II). Since the clinical efficacy of 3DU is still under evaluation, it appeared prudent to conduct an antitumor comparison of these two compounds to determine whether preclinical advantages exist for CPE-C that might indicate that this compound was worth continued development toward clinical trial. Because the existing 3DU preclinical antitumor data are quite old, and several of the currently used tumor models were not available when 3DU was originally studied, we conducted direct comparison evaluations of 3DU and CPE-C in four preclinical tumor models (Table IV).

In the MX-1 human mammary solid tumor xenograft system, once again (see Table III) CPE-C was very effective, giving complete inhibition of tumor growth relative to untreated controls. 3DU was inactive according to established NCI criteria.[27] Against the human LOX melanoma xenograft, 3DU was, again, inactive with a 3 $\log_{10}$ increase in tumor burden observed at the end of treatment. In contrast, CPE-C was active, producing an ILS of 80% at an optimum dose of 2.25 mg/kg on the QD 1–9 treatment schedule. This resulted in a 2 log tumor cell kill[28] and, therefore a 5 log difference in tumor burden between CPE-C and 3DU as the result of drug treatment. CPE-C was about 100 times more potent than 3DU in both xenograft experiments.

Because CPE-C was so active against ara-C resistant P388 leukemia (P388/ara-C, Table II) and the reported activity of CPE-C and 3DU against L1210/ara-C,[12,26] the two compounds were directly compared in the sensitive and ara-C resistant L1210 models. Although 3DU was active in the standard L1210 leukemia system (80% ILS at 100 mg/kg), CPE-C was significantly better in this "head-to-head" comparison, producing multiple long-term survivors at 1.5 mg/kg, consistent with our earlier experiments (Table II). Against L1210/ara-C, however, both 3DU and CPE-C were very active, giving multiple 60-day survivors at more than one dose. In this model there was essentially no difference between CPE-C and 3DU other than the usual greater potency of CPE-C.

Antiviral Activity. Carbodine (1) was shown to be very effective against several viruses and in particular influenza viruses.[6b,15] This activity, however, did not carry over to the in vivo situation. Slight carbodine activity was noted against rhinovirus 1A,[15] respiratory syncytial virus (RSV),[29] and two other RNA viruses. Good activity, however, was noted against several DNA viruses—herpes simplex type 1 (HSV-1), HSV-2, and vaccinia.[6b,15] Carbodine was found to be more potent against a strain of the HSV-1 virus deficient in its ability to induce thymidine kinase in the host cell (TK−) than a strain that induces this enzyme (TK+).[18] However, it is possible that experimental parameters (e.g. host cell type, virus strain) may influence carbodine's activity against HSV-1 (TK−).[6b] Because carbodine (1) had activity against a number of viruses in vitro, similar studies were undertaken with CPE-C (2).

CPE-C shows significant activity in several DNA viral systems as measured by its ability to inhibit viral cytopathogenic effects (Table V). CPE-C potency and activity [measured by its virus rating (VR)] are greater than those of arabinosyl adenine (ara-A) against both the TK− and TK+ strains of HSV-1. This confirms the high potency of CPE-C against HSV-1 (TK−) reported earlier by De Clercq et al.[18] Acyclovir is more active than CPE-C against the TK+ strain, but it is inactive against the non enzyme inducing strain (TK−) since thymidine kinase is required to activate acyclovir. A similar profile is observed against HSV-2 (Table V). A fourth DNA virus, vaccinia, is very sensitive to CPE-C. The carbocyclic nucleoside is not only more active than the positive control compound, ara-A, but also is ca. 100 time more potent against vaccinia virus. Table VI compares the activity of CPE-C with DHPG against cytomegalovirus and varicella-zoster by using reduction of virus yield and percent plaque reduction as respective endpoints. Activity is observed for both compounds, but the positive control (DHPG) is superior in both instances.

CPE-C has significant activity against a spectrum of RNA viruses in vitro (Table VII). Virus ratings greater than two are obtained against vesicular stomatitis virus, Punta Toro virus, and the Hong Kong influenza virus. The

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Table V. Evaluation of CPE-C against DNA Viruses[a,b]

virus	strain	CPE-C VR	CPE-C ID_{50}, μg/mL	positive control compound	positive control VR	positive control ID_{50}, μg/mL
herpes simplex type 1	E-377 (TK+)[c]	3.8	0.3	ara-A	1.8	13.6
				acyclovir	6.7	0.7
herpes simplex type 1	HF (TK−)[c]	3.8	0.6	ara-A	3.3	2.1
		2.7	1.3		2.9	1.9
herpes simplex type 2	MS	2.3	2.7	ara-A	1.3	49.9
				acyclovir	4.5	5.3
vaccinia	Lederle CA	4.6	0.1	ara-A	3.1	9.8

[a] The antiviral activity of each compound is expressed as a virus rating (VR), and the potency is given as an ID_{50}. The VR is a weighted measurement of antiviral activity that takes into account both the degree of inhibition of virus-induced cytopathogenic effects and the degree of cytotoxicity produced by the test compound (see the Experimental Section). The ID_{50} is the concentration of the test compound in μg/mL required to inhibit the virus-induced cytopathogenic effects by 50%. [b] Vero cells (African green monkey kidney cells) were host cells in all experiments. [c] HSV-1 strain E-377 induces thymidine kinase in host cells. Strain HF does not induce this enzyme.

Table VI. Evaluation of CPE-C against Cytomegalovirus and Varicella-Zoster Virus

concn, μg/mL	cytomegalovirus[a] reduction in virus yield, log PFU/mL[b] CPE-C	DHPG[c]	vericella-zoster[a] plaque reduction,[b] % CPE-C	DHPG
320	5.3[d]	5.3[e]	36[d]	100[d]
100	2.0[e]	5.3[f]	41[e]	100[f]
32	2.0[e]	5.3	36[e]	85
10	1.9[e]	3.8	33[f]	69
3.2	1.5[e]		33	46
1.0	1.4[e]	1.1	36	44
0.32	1.3[f]		33	
0.1	0.8		21	
0.032			3	
0.01			0	

[a] CMV strain AD 169. The host line is MRC5 (diploid human embryonic lung cells). VZV isolate DM625. The host cell line is Huf (human diploid foreskin cells in monolayer culture). [b] See the Experimental Section. [c] 9-[(1,3-Dihydroxy-2-propoxy)methyl]-guanine. [d] Slightly toxic based on gross cell morphology. [e] Very slightly toxic. [f] This dose and all lower doses were nontoxic.

utility of these activities, however, is moderated by low therapeutic index values. By contrast, this does not appear to be a problem in the Japanese encephalitis virus system, where CPE-C has a VR of 2.4, with a greater potency and higher therapeutic index than Ribavirin, the positive control compound. Activity was not seen against Rhinovirus 1A. No anti-HIV (retroviral) activity was observed using the Mitsuya and Broder assay,[30] and potent cytotoxicity to the ATH8 host cell line was noted.

CPE-C antiviral activity is tempered by observed cellular toxicity and the knowledge that the compound has antitumor properties that are cytotoxic in nature. Morphological changes, although slight, were noted upon microscopic examination of CPE-C-treated cells during antiviral experiments. This led to a study of the effect of CPE-C on Vero cells (Table VIII). Viable cells were reduced by 50% even at 0.1 μg/mL concentrations. This effect plateaued with ca. one-third of the viable cells remaining at all higher concentrations evaluated. H.Ep-2 cells, a line of tumor origin, was especially sensitive to the cytotoxic effects of CPE-C and could not be used as an antiviral host cell line.

Conclusion

Enantiomerically pure cyclopentenylcytosine (CPE-C) has been prepared for antitumor and antiviral evaluation. This compound has significant activity against model tumor systems in vivo and viruses in vitro. The potent cytotoxicity of CPE-C, howver, makes the development of the antitumor aspects of the compound the more attractive possibility initially.

Experimental Section

All chemical reagents were commercially available. Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Proton and ^{13}C NMR spectra were obtained on a Varian XL-200 instrument. Proton chemical shifts are expressed as δ values with reference to Me_4Si. For ^{13}C NMR, the peak positions were determined by reference to dioxane (δ 67.3). Specific rotations were measured in a 4-dm cell with a Perkin-Elmer Model 241 polarimeter. Positive-ion fast atom bombardment (FAB) mass spectra were obtained on a VG 7070D mass spectrometer that was equipped with a FAB ion source. The sample was dissolved in a glycerol matrix, and ionization was effected by a beam of xenon atoms derived by neutralizing xenon ions accelerated through 8.6 kV. Normal-phase column chromatography was run on silica gel (J. T. Baker 60–200 mesh), and analytical TLC was performed an Analtech Uniplates silica gel GF with the solvents indicated for the individual experiments. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.

Ethyl 3-[(Ethoxycarbonyl)amino]-3-oxopropanoate. This material was prepared from malonyl chloride and urethane in 44% yield according to a published procedure, mp 57–58 °C (lit.[20] mp 57–58 °C).

3-Ethoxypropenoyl Isocyanate (4). This compound was prepared from 3-ethoxy-2-propenoyl chloride[31,32] as described in the experimental procedure for **7**. It was used immediately.

Ethyl 3-Ethoxy-2-[[(ethoxycarbonyl)amino]carbonyl]-2-propenoate (5, Holy's Reagent). This compound was prepared in 57% yield according to Holy's procedure by reacting ethyl 3-[(ethoxycarbonyl)amino]-3-oxopropanoate with triethyl orthoformate. The solid obtained had a melting point (mp 127–129 °C) higher than the reported literature value (mp 98–99 °C).[20a]

(−)-*N*-[[[(1*R*,4*R*,5*S*)-3-[(Benzyloxy)methyl]-4,5-(isopropylidenedioxy)-2-cyclopentenyl]amino]carbonyl]-3-ethoxy-2-propenamide (7). 3-Ethoxypropenoyl chloride (23.5 g, 175 mmol) in benzene (300 mL) was added dropwise to a vigorously stirred suspension of silver isocyanate (65.5 g, 430 mmol), which was predried by azeotropic distillation in benzene. The temperature during the addition was held to 10 °C, and after 30 min the suspension was filtered. The silver chloride and excess of silver isocyanate were washed with benzene (2 × 100 mL), and the combined filtrate containing the 3-ethoxypropenoyl isocyanate (4) was used immediately. A benzene solution of freshly prepared amine **3b** [from 41 g (138 mmol) of the azide **3a**] was added dropwise to the benzene solution of 3-ethoxypropenoyl isocyanate while the temperature was maintained at 10 °C. The pale yellow solution formed was stirred for 30 min at the same temperature and after that time the solvent was removed under reduced

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Table VII. Evaluation of CPE-C against RNA Viruses[a,b]

virus	strain	host cell[c]	CPE-C VR	CPE-C ID_{50},[d] μg/mL	CPE-C TI[e]	positive control compound	positive control VR	positive control ID_{50}, μg/mL	positive control TI
vesicular stomatitis	Indiana	L929	2.4	0.57	0.6	3-deazaaristeromycin	2.1	4.3	2.3
			3.0	0.25	0.4		3.0	2.0	1.6
yellow fever	Asibi	vero	0.9	5.05	0.1	selenazole	2.4	1.8	0.6
Japanese encephalitis	Najayama	vero	2.4	0.10	3.1	ribavirin	2.4	3.0	1.1
Punta Toro	Adames	vero	1.5	1.01	0.3	ribavirin	1.8	20.1	5.0
influenza (Hong Kong)	A_2/Aichi/2/68	MDCK	2.4	18.2	ND	ribavirin	3.6	18.7	ND

[a] See Table I for definitions. [b] Data obtained from the USAMRIID antiviral testing program[31] except for influenza, which was obtained under an NCI purchase order (see the Experimental Section). USAMRIID virus rating values, calculated by the method of Sidwell and Huffman[33] were converted to Ehrlich values[32] for consistency by multiplication by three. [c] L929 cells are normal mouse fibroblasts. MDCK cells are Madin-Darby canine kidney cells. [d] Concentration of the drug that causes a 50% reduction in virus replication. [e] Therapeutic index equals minimum toxic drug concentration (concentration causing 50% reduction in percent survival of host cells) divided by the ID_{50} for the drug.

Table VIII. Drug Cytotoxicity As Determined by Viable[a] Cell Counts of Vero Cell Monolayers Treated with CPE-C for 4 Days

drug concn, μg/mL	redn in no. of viable cells after drug treatment, %	drug concn, μg/mL	redn in no. of viable cells after drug treatment, %
320	67	0.1	50
100	66	0.032	3
10	64	0.01	0
1.0	61		

[a] Trypan blue dye exclusion method.

pressure (45 °C) to give 60 g of **7** as a semisolid material. After this material was dissolved in ether (250 mL), the cloudy solution was filtered (Celite) and the clear filtrate was further diluted with ether (75 mL) and petroleum ether (150 mL). The solution was stirred overnight at 5 °C, after which time a yellow solid precipitated out. This solid was collected and dried to give 49 g (85%) of intermediate **7**, mp 87–90 °C.

(1*R*,4*R*,5*S*)-(−)-1-[3-[(Benzyloxy)methyl]-4,5-(isopropylidenedioxy)-2-cyclopentenyl]-5-(ethoxycarbonyl)uracil (8a). Lindlar's catalyst (14 g) was added to a solution of the azide **3a** (35 g, 120 mmol) in ethanol (350 mL). The mixture was hydrogenated at atmospheric pressure for 2 h. Purging with fresh hydrogen was performed at 0.5-h intervals. The reaction mixture was filtered, and Holy's reagent (**5**, 35 g, 140 mmol) was added to the filtrate, which was heated at reflux for 40 min. Triethylamine (35 g, 350 mmol) was added, and the solution was heated at reflux for 18 h. The solvent was removed, and the gummy residue was dissolved in ethanol (175 mL). Ether (640 mL) was added, and the mixture was cooled to −10 °C and filtered. The collected solid was washed with cold ether (2 × 70 mL) and air-dried to give the uracil analogue **8a** (33 g, 64%) as a white crystalline solid: mp 167–168 °C; $[\alpha]^{23}_D$ −70° (*c* 0.5, ethanol); NMR ($CDCl_3$) δ 1.34 (s and t, *J* = 7.0 Hz, 6 H, CH_2CH_3, and isopropylidene methyl), 1.43 (s, 3 H, isopropylidene methyl), 4.28 (q, *J* = 7.0 Hz, 2 H, CH_2CH_3), 4.30 (br s, 2 H, H-6′$_{a,b}$), 4.60 (s, 2 H, OCH_2Ph), 4.64 (d, *J* = 6.0 Hz, 1 H, H-5′), 5.27 (d, *J* = 6.0 Hz, 1 H, H-4′), 5.40 (s, 1 H, H-1′), 5.70 (s, 1 H, H-2′), 7.34 (m, 6 H, NH, Ph), 8.05 (s, 1 H, H-6). Anal. ($C_{23}H_{26}N_2O_7$) C, H, N.

(1*R*,4*R*,5*S*)-(−)-1-[3-[(Benzyloxy)methyl]-4,5-(isopropylidenedioxy)-2-cyclopentenyl]-5-uracilcarboxylic Acid (8b). A solution of lithium hydroxide monohydrate (7.3 g, 170 mmol) in water (70 mL) was added to a suspension of ester **8a** (33 g, 75 mmol) in methanol (200 mL). The mixture was stirred at ambient temperature for 2 h. The resulting solution was cooled to 0 °C and added to a cold suspension of Dowex 50W-X2 (H^+) resin column (450 g) and eluted with methanol (3 L). The eluate was concentrated to ca. 300 mL, and the acid crystallized on standing. The solid was collected, washed with cold ether (2 × 75 mL), and air-dried to give 22 g (71%) of acid **8b** as a white crystalline solid: mp 208–209 °C; $[\alpha]^{23}_D$ −65° (*c* 0.5, 1 N NaOH). This material was used as such in the following step.

(1*R*,4*R*,5*S*)-(−)-1-[3-[(Benzyloxy)methyl]-4,5-(isopropylidenedioxy)-2-cyclopentenyl]uracil (9a). Method A. The crude acid **8b** (8.5 g, 21 mmol) was added to a hot (170 °C) mixture of quinoline (32 mL), 1,10-phenanthroline (19 g, 110 mmol), and cuprous oxide (9.94 g, 66 mmol). The resulting mixture was stirred for 15 min at 170 °C and cooled. Ethyl acetate (240 mL) was added, and the mixture was filtered through Celite. The solvent was removed under vacuum, and the dark oil obtained was dissolved in fresh ethyl acetate (240 mL), cooled to 0 °C, and washed with cold 0.5 N HCl (2 × 200 mL). The organic phase was further washed with saturated sodium bicarbonate solution and water and was dried over Na_2SO_4. The solvent was removed under vacuum, and the residual gum was purified by column chromatography on silica gel (240 g) by eluting first with ether and then with ethyl acetate. The product-containing fractions were combined and evaporated to give the protected uracil analogue **9a** (10.8 g, 71%) as an off-white foam. A portion of this material was recrystallized from ether to give pure compound: mp 100–102 °C; $[\alpha]^{23}_D$ −26.8° (*c* 0.46, $CHCl_3$); NMR ($CDCl_3$) δ 1.34 and 1.43 (s, 6 H, isopropylidene CH_3), 4.23 (br s, 2 H, H-6′$_{a,b}$), 4.60 (m, 3 H, H-5′ and CH_2Ph), 5.23 (d, *J* = 5.5 Hz, 1 H, H-4′), 5.36 (s, 1 H, H-1′), 5.61 (s, 1 H, H-2′), 5.68 (d, *J* = 8.0 Hz, 1 H, H-5), 7.00 (d, *J* = 8.0 Hz, 1 H, H-6), 7.34 (br s, 5 H, Ph), 10.05 (s, 1 H, NH). Anal. ($C_{20}H_{22}N_2O_5$) C, H, N.

Method B. Intermediate **7** (48.5 g, 117 mmol) was dissolved in dimethylformamide (250 mL). Concentrated ammonium hydroxide (1.5 L) was added, and the resulting suspension was refluxed for 4 h while a stream of ammonia gas was bubbled through the suspension. The resulting mixture was diluted with water (300 mL) and cooled to room temperature, after which it was extracted with a mixture of ether and ethyl acetate (1:1, 6 × 300 mL). The organic extracts were combined, washed with water, dried ($MgSO_4$), charcoaled, and reduced to dryness under vacuum. The solid residue was dissolved in ether (500 mL), and the solution was filtered (Celite). The volume was reduced to ca. 250 mL, and the solution was kept in the refrigerator overnight. The resulting crystalline solid was washed with cold ether and petroleum ether to give 27 g (62%) of **9a**, mp 98–100 °C, which was identical in all respects with the material obtained under Method A.

(1*R*,4*R*,5*S*)-(−)-1-[3-[(Benzyloxy)methyl]-4,5-(isopropylidenedioxy)-2-cyclopentenyl]-4-thioxouracil (9b). Lawesson's reagent[22] (3.6 g, 8.9 mmol) was added to a solution of the protected uracil **9a** (4.5 g, 12.2 mmol) in hexamethylphosphorus triamide (15 mL), and the resulting mixture was heated at 100 °C for 2.45 h. After being cooled to room temperature, the solution was extracted with a mixture of ethyl acetate (100 mL) and saturated sodium bicarbonate (100 mL). The organic phase was separated. The aqueous phase was reextracted with ethyl acetate, and the combined organic extracts were washed with brine (5 × 75 mL) and dried (Na_2SO_4). The solvent was removed to give a brown foam, which was chromatographed on silica gel (250 g) with petroleum ether and ethyl acetate (gradient: 9:1, 8:2, 7:3) as the eluant mixture. The fractions that contained product were combined and evaporated to give 3 g (64%) of the desired compound **9b** as a yellow foam. This material was used as such in the following step.

(1*R*,4*R*,5*S*)-1-[3-[(Benzyloxy)methyl]-4,5-(isopropylidenedioxy)-2-cyclopentenyl]cytosine (10). Liquified ammonia (45 mL) was added to a cold (−40 °C) solution of the thiouracil analogue **9b** (2.8 g, 7.3 mmol) in methanol (45 mL). The mixture was heated to 60 °C in a pressure vessel for 18 h and then evaporated to dryness. The brown residue was purified by column chromatography on silica gel (40 g) with a chloroform/methanol mixture (9:1) as eluant to give a brownish foam. This foam was

dissolved in hot ethyl acetate, charcoaled, and filtered while hot. The filtrate was cooled to −10 °C, and the solid formed was collected, washed with ether, and dried to give 1.09 g (41%) of **10** as a white solid: mp 154–155 °C; $[\alpha]^{23}_D$ −56.7° (*c* 0.6, ethanol). Anal. ($C_{20}H_{23}N_3O_4$) C, H, N.

In a scale-up preparation with 10 g of **9b** as starting material, a 64% yield of the desired **10** was obtained.

(1*R*,4*R*,5*S*)-1-[3-(Hydroxymethyl)-4,5-dihydroxy-2-cyclopenten-1-yl]cytosine (2, CPE-C). Boron trichloride (9.6 mL of a 1 M solution in methylene chloride, 9.6 mmol) was added to a cold (−78 °C) solution of intermediate **10** (1.2 g, 3.3 mmol) in methylene chloride (28 mL). The solution was stirred at −78 °C for 2.5 h and allowed to warm up to 0 °C. Methanol (20 mL) was added, and the solvents were removed in vacuo. Additional methanol (20 mL) was added and evaporated again to give a residual gum, which was dissolved in water (10 mL). The aqueous solution was extracted with ether (4 mL) and applied to a Dowex 50W-X2 (H^+) column (12 mL). The column was first eluted with water and then with 2 N ammonium hydroxide. The fractions containing the product were combined, treated with charcoal (120 mg), filtered, and lyophilized to a foam. Ethanol (12 mL) was added to the foam and heated to 65 °C. The resulting solution was allowed to cool, and the desired compound **2** (0.49 g, 62%) crystallized as a white solid, mp 135–139 °C. A second recrystallization afforded an analytical sample of **2**: mp 138–141 °C; $[\alpha]^{23}_D$ −104.5° (*c* 0.13, H_2O), NMR (D_2O) δ 4.14 (t, *J* = 6 Hz, 1 H, H-5′), 4.30 (s, 2 H, H-6′$_{a,b}$), 4.60 (d, *J* = 6 Hz, 1 H, H-4′), 5.45 (m, 1 H, H-1′), 5.80 (d, *J* = 1.5 Hz, 1 H, H-2′), 6.00 (d, *J* = 8 Hz, 1 H, H-5), 7.44 (d, *J* = 8 Hz, 1 H, H-6); FAB mass spectrum, *m/z* (relative intensity) 332 (MH + glycerol, 17), 240 (MH^+, 88), 112 (b + 2H, 100). Anal. ($C_{10}H_{13}N_3O_4 \cdot 0.25H_2O$) C, H, N.

Antitumor Evaluations. In vivo antitumor tests were conducted under contract to the NCI. They were directed by Dr. Daniel Griswold, Jr., Southern Research Institute, Birmingham, AL, Dr. David Houchens, Battelle Columbus Laboratories, Columbus, OH, and Mr. Alan Shefner, IIT Research Institute, Chicago, IL. Tests were carried out according to establish NCI protocols.[27] Tumor cells were implanted at the specified sites, and treatment was initiated 24 h later. Drugs were administered intraperitoneally in physiologic saline vehicle, and their effects were evaluated at several dosage levels in each experiment. Life-span experiments were terminated on day 30 unless otherwise noted. Subrenal capsule assays were evaluated on day 11. Long term survivors are included in the %ILS calculations. When activity was observed in an initial test, a minimum of one confirmatory test was conducted, normally at a different testing laboratory. In the instance of the direct comparison of CPE-C and 3DU against L1210/ara-C, only one experiment was conducted because of existing 3DU data.

Antiviral Evaluations. Antiviral evaluations were carried out under NIH purchase order 263-MD-610174 at Southern Research Institute, Birmingham, AL, under the direction of Dr. William Shannon and Ms. Gussie Arnett.

Cytopathogenic Inhibition Test. Mammalian cells were pregrown as monolayer cultures in wells of COSTAR 96-well tissue culture plates with suitable cell culture media. Stock viruses were pretitered and diluted in cell culture medium to yield 32 $CCID_{50}$ (cell culture infectious dose, 50%) units per 0.1 mL. Antiviral assays were designed to test a minimum of seven concentrations of each compound from cytotoxic to noncytotoxic levels against each of the challenge viruses in triplicate in microtiter plate wells containing suitable host cell monolayers. To each of the replicate cell cultures was added 0.1 mL of the test drug solution and 0.1 mL of virus suspension. Cell controls containing cells + medium, virus controls containing cells + medium + virus, and drug cytotoxicity controls containing cells + each drug in medium were run simultaneously with the test samples assayed in each experiment. The covered plates were incubated at 37 °C in a humidified atmosphere containing 2% CO_2 and then examined microscopically for virus-induced cytopathogenic effects (CE) at 3–4 days postvirus inoculation. Antiviral activity was determined by calculating the degree of inhibition of virus-induced CE in drug-treated, virus-infected cell cultures by means of a virus rating (VR). The VR is a standard weighted measurement of antiviral activity taking into account both the degree of CE inhibition and drug cytotoxicity and is determined by a modification of the method of Ehrlich et al.[32] The VR was calculated as 0.1 of the sum of the numerical differences between the recorded CE grade of each test well and that of the corresponding virus control in the culture plate. Drug cytotoxicity was determined by gross morphological changes in the cell monolayers observed by microscopic examination. Numerical differences between the reading of test wells containing a drug concentration which was partially cytotoxic (cell monolayer intact) and their corresponding virus controls were halved.

Experience shows that a VR of 1.0 or greater is indicative of significant antiviral activity with a high degree of reproducibility in confirmatory in vitro tests. Compounds with a VR of 1.0 or greater are therefore considered active. A compound with a VR of 0.5–0.9 is considered to have possible or marginal activity, and any compound with a VR of less than 0.5 is considered to be inactive in this test system. The ID_{50} (dose required to inhibit virus-induced CE by 50%) is also determined.

Cytomegalovirus (CMV) Yield Reduction Assay. Subconfluent monolayers of MRC5 cells, grown in 35-mm wells of Falcon six-well tissue culture plates, were rinsed with phosphate-buffered saline (PBS) and exposed to 0.5 mL of virus (diluted in Eagle's MEM + 2% fetal bovine serum to a multiplicity of infection of 0.2 PFU/cell) for 1.5 h at 37 °C. The virus inocula were removed after adsorption, and the infected cell layers were rinsed with PBS. Duplicate infected cell layers were fed with 2.0-mL aliquots of each drug concentration (dissolved and diluted in MEM + 2% FBS). Untreated, infected controls and cell controls were fed with medium. Uninfected drug-treated cell cultures served as cytotoxicity controls. The cultures were incubated at 37 °C in a humidified atmosphere containing 2% CO_2. On day 3 postinfection, the fluids were aspirated and replaced with fresh drug and/or medium. The cell monolayers were examined microscopically on day 6 for viral CE and harvested as follows: The cultures were frozen and thawed to disrupt the cells. The cellular material was scraped into the ambient medium, and the contents from replicate cultures were pooled, dispensed into cryotubes, and stored in liquid nitrogen.

The harvested samples were thawed and titrated for infectious virus yield by the following plaque assay procedure. Subconfluent cell monolayer cultures pregrown in 16-mm 12-well tissue culture plates were rinsed with PBS and exposed to 0.25-mL aliquots of harvested virus suspension for 1.5 h at 37 °C in a humidified atmosphere of 2% CO_2 in air. The virus inocula were removed after adsorption, and the infected cell layers were rinsed with PBS. Triplicate virus-infected cell layers were overlaid with each drug concentration contained in 1 mL of medium (MEM + 2% FBS in 0.25% agarose). Untreated virus-infected cell controls, drug cytotoxicity controls (sham-infected cell monolayer cultures overlaid with drug-containing medium), and cell culture controls were included. The tissue culture plates were incubated at 37 °C in the CO_2 incubator for 5–6 days, until discrete well-defined foci (plaques) of viral CE were formed. The cell layers were fixed with 10% buffered formalin and stained with 0.03% methylene blue. The plates were examined microscopically, and the plaques were counted under low magnification. Antiviral activity was measured by comparing the mean number of plaques from triplicate drug-treated, virus-infected cell cultures with the mean number of plaques in the untreated virus control cultures. Any reduction in the mean number of plaques present in drug-treated, virus-infected cell cultures, when compared with the mean number of plaques in the virus-infected control cultures, was expressed as percent reduction. The minimum drug concentration that reduced the mean plaque number by 50% (ID_{50}) was calculated by using a regression analysis program for semilog curve fitting.

Varicella-Zoster Virus (VZV) Plaque Reduction Assay. The plaque reduction assay procedure for VZV was the same as that used to titrate samples from the CMV yield assay, except that the VZV virus inocula were not removed after a 1-h virus adsorption period. Agarose was omitted from the drug-containing overlay medium. The VZV-infected human foreskin cell monolayer cultures were fed with fresh drug and medium after 72-h incubation and returned to the incubator. The discrete foci of CE (plaques) were counted 6 days post virus inoculation.

Quantitative Drug Cytotoxicity Study. Vero cell monolayer cultures were grown in 35-mm diameter wells of Falcon six-well tissue culture plates at 37 °C. Duplicate cell cultures were treated

with each concentration of CPE-C in liquid cell culture medium. The plates were returned to the CO_2 incubator at 37 °C. After four days incubation, the cytotoxicity was measured by performing viable cell counts (utilizing the trypan blue dye exclusion method) on the drug-related and untreated control cell cultures.

U.S. Army antiviral tests were carried out at Southern Research Institute under standard protocols developed by the Department of Antiviral Studies, U.S. Army Medical Research Institute of Infectious Disease.[33]

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Synthesis and Bacterial DNA Gyrase Inhibitory Properties of a Spirocyclopropylquinolone Derivative

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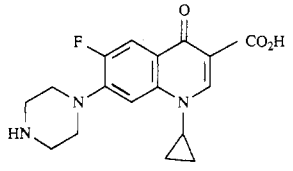
A novel conformationally restricted 1-cyclopropylquinolone (1) that incorporates structural features of both ofloxacin and ciprofloxacin has been prepared. Compound 1 was found to be a DNA gyrase inhibitor having potency similar to ofloxacin but less than ciprofloxacin. The cellular inhibitory and in vivo antibacterial potencies of 1 were found to be less than those of the two reference agents.

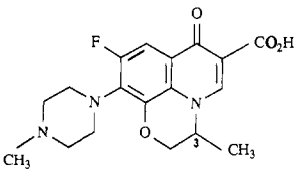
The exceptional in vitro antibacterial properties of ciprofloxacin (CIP) relative to many other quinolones can be attributed to its 1-cyclopropyl substitution.[1-4] Those quinolones that have ethyl (e.g. norfloxacin),[5] aryl (e.g. difloxacin),[6] or methylamino (e.g. amifloxacin)[7] appendages at position 1 are generally less potent in vitro, especially vs Gram-negative bacteria.

Quinolones exert their antibacterial effect via inhibition of bacterial DNA gyrase, an enzyme essential for procaryotic DNA replication.[2-4] While the molecular interaction of quinolones with the gyrase enzyme is not fully understood, quinolones have been reported to bind to single-stranded DNA but not to gyrase or double-stranded DNA.[8,9]

As part of a study into the conformational requirements of 1-cyclopropylquinolones with respect to their role as inhibitors of gyrase function, we have prepared a novel, conformationally restricted analogue (1) of CIP.[10] For 1, the quaternary carbon of the cyclopropyl ring is incorporated into an additional ring, which ultimately links positions 1 and 8 of the quinolone. The resulting benzoxazine structure can also be considered a spirocyclopropyl variant of the clinically useful quinolone, ofloxacin (OFL).[2]



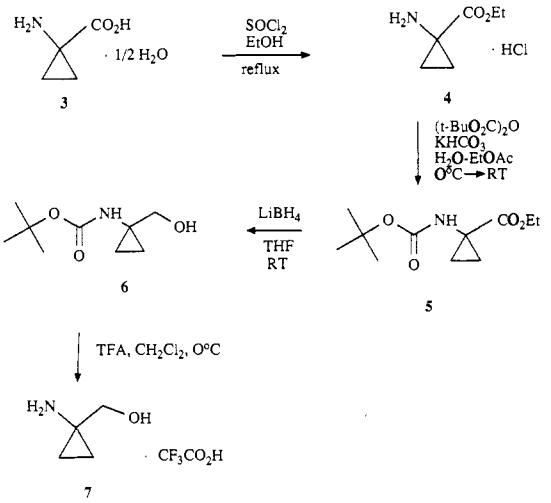
Ciprofloxacin(CIP)



Ofloxacin(OFL)

An additional analogue, **2**,[11] was prepared to assess the biological effect upon disubstitution at position 3 of OFL. Compound **2** lacks the pseudo-π interactions[12] the cyclopropyl group of 1 might have with the quinoline ring or biochemical target but maintains a spatial requirement at position 3 more similar to 1 than OFL. Recent studies have shown that the *S*-(-) enantiomer of OFL (the methyl group orientated β as normally drawn) is a 30-fold more potent *E. coli* gyrase inhibitor than its mirror image.[13]

Scheme I



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(10) After submission of this manuscript, a synthesis of 1 was published utilizing similar methodology: Jpn. Pat. 62-198685. No quantification of biological activity was given.
(11) Subsequent to the completion of this work, a report appeared describing the synthesis of **2** by a similar procedure: Grohe, K.; Schriewer, M.; Zeiler, H.-J.; Metzger, K. G. EP 206,076. Grohe, K.; Schriewer, M.; EP 206,101. No quantification of biological activity was provided.

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