0.2 mmol/kg of the test agent. The number of days the mice survived beyond that of mice treated with phosphate buffer containing 1% dimethyl sulfoxide was used as a measure of biological activity.

Measurement of Methanol Generation. The generation of methanol was assayed by placing 2.2 mL aliquots of a 1:200 dilution of aged solutions of the composition described above into a Gilson Oxygraph. Twenty microliters of *Pichia pastoris* alcohol oxidase (666 units/mL) was then added and the resultant O_2 consumption was used as a measure of methanol content.

Antineoplastic Activity. The ascites cell forms of leukemias L1210 and P388 were obtained from the Frederick Cancer Research Facility DCT Tumor Repository of the National Cancer Institute and Sarcoma 180 ascites cells were obtained from stocks available at the Yale Comprehensive Cancer Center; these lines were maintained by serial passage in tissue culture. Every 8 weeks, the tumor cells were injected intraperitoneally into five donor mice (CD_2F_1) 8–10 weeks of age and were allowed to grow for 7 days. The peritoneal fluid was withdrawn and the suspension centrifuged for 5 min (1600g). The supernatant was decanted and 1 × 10^5 cells/mL were seeded in 10 mL of RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% glutamine, and once again maintained in culture. For the assay, 0.1 mL of the cell suspension containing 10^5 cells (10^6 cells in the case of the P388 leukemia and Sarcoma 180) was injected into each recipient mouse. The test compounds were administered over a wide range of dosage levels in the case of the L1210 leukemia and for compound **5** at 60 mg/kg for Sarcoma 180 and the P388 leukemia, beginning 24 h after tumor implantation, once daily for 6 consecutive days. Compound **3** was also administered as single doses as noted in Table I. All drugs were administered intraperitoneally as solutions in dimethyl sulfoxide in a volume not exceeding 0.025 mL. For any one experiment, animals were distributed into groups of five mice of comparable weight and maintained throughout the course of the experiment on Purina Laboratory Chow pellets and water ad libitum. Control tumor-bearing mice given comparable volumes of vehicle were included in each experiment. Mice were weighed during the course of the experiments, and the percentage change in body weight from onset to termination of therapy was used as an indication of drug toxicity. Determination of the sensitivity of these neoplasms to these agents was based upon the prolongation of survival time afforded by the drug treatments.

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Monophosphoric Acid Diesters of 7β-Hydroxycholesterol and of Pyrimidine Nucleosides as Potential Antitumor Agents: Synthesis and Preliminary Evaluation of Antitumor Activity

Yu-hua Ji, Christiane Moog, Gaby Schmitt, Pierre Bischoff,† and Bang Luu*

Laboratoire de Chimie Organique des Substances Naturelles, URA CNRS No. 31, 5 rue Blaise Pascal, 67084 Strasbourg, France, and Laboratoire de Recherches en Immunologie, Institut d'Hématologie et d'Immunologie, 1 Place de L'Hôpital, 67091 Strasbourg, France. *Received February 7, 1989*

7β-Hydroxycholesterol, which has been shown to be selectively cytotoxic toward tumor cells cultered in vitro, was converted into the corresponding water-soluble phosphoric acid ester and linked to a pyrimidine nucleoside such as 5-fluoro-2′-deoxyuridine or 2′-deoxyuridine. 2-Chlorophenyl phosphorodichloridate (**3**), without activation, was used directly to phosphorylate the protected oxygenated sterol. The intermediate phosphorylated the 5′-OH group of nucleoside selectively, leading to compounds **1a** and **1b** after deprotection. These compounds were screened for their antiproliferative activity toward EL-4 murine leukemia cells in vitro and for their antitumor activity against the mice bearing Krebs II ascitic carcinoma in vivo.

Introduction

Sterols and triterpenes bearing several oxygen functions have demonstrated a wide variety of biological activities[1,2] expressed, in particular, by the inhibition of several steps in the biosynthesis of cholesterol.[3,4] Our laboratory has long been interested in the selective cytotoxicity of these compounds. A series of polyoxygenated sterols and triterpenes either isolated from traditional antitumor remedies or synthesized have been shown to be much more toxic toward tumor cells than toward normal ones.[5,6] Another important effect of this class of compounds is their action on the cell membrane.[7-9] This effect is different from that of some classical antitumor drugs such as nucleoside analogues, whose action focuses mainly on the cell cycle. 7β-Hydroxycholesterol (7β-OHC), one of these oxysterols, has been intensively studied because of its high activity and its relatively easy synthesis.[10] Unfortunately, the low water solubility of 7β-OHC makes its use difficult for in vivo studies. For this reason, we have used more water-soluble derivatives. The sodium bis(hemisuccinate) of 7β-hydroxycholesterol (BHS-7β-OHC) has been synthesized, and its antitumor activity on mice bearing Krebs II ascitic carcinoma gave very encouraging results.[11] However, its moderate water-solubility (<2%) did not satisfy our requirements in further biological assays and this led us to undertake the synthesis of more elaborate

† Institut d'Hematologie et d'Immunologie.

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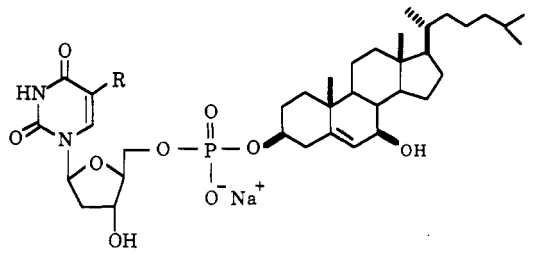
Scheme I

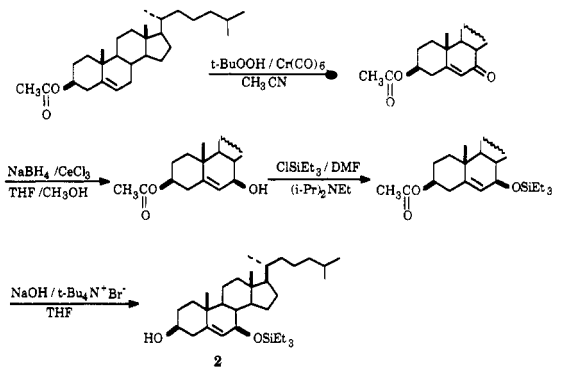


R = F; **1a**
R = H; **1b**

derivatives: the phosphodiesters of oxysterols conjugated with other hydrophilic molecules, for example, nucleosides.

In comparison, 5-fluoro-2′-deoxyuridine (5F-dUrd), a clinically useful prodrug of 5-fluorouracil,[12-14] suffers from some problems such as toxic side effects and, most relevant to our work, its very short half-life in plasma owing to its rapid catabolism into biologically inactive compounds.[15-17] The drug resistance of some tumor cells is another challenging problem which is caused by lack of thymidine kinase, an enzyme responsible for converting 5F-dUrd to the actual cytotoxic metabolite FdUMP. Initial studies of the esters of 5F-dUrd and the corresponding nucleoside 5′-phosphate showed that they were not suitable as chemotherapeutic agents. Indeed, these derivatives had a poor ability to penetrate into cell membranes and were rapidly dephosphorylated to their parent nucleoside.[18-20] However, it has also been demonstrated recently that lipophilic derivatives of 5F-dUrd may overcome these drawbacks. In fact, the attempts to administer 5F-UdR as a neutral 5′-phosphate triester, phosphoramidate, or monocharged 5′-diester have met with some success.[21-24] By preparing the 5′-(3-*sn*-phosphatidyl)-5-fluorouridine, Shuto et al. have shown that the nontoxic phosphatidyl residue acting as carrier moiety could protect the drug from inactivation by enzymes and that the resulting conjugated compound possesses a high affinity for cell membranes and penetrates easily into cells.[24] This compound resulted in a significant increase in the life span of mice bearing ip-transplanted P388 leukemia, being more effective than the parent nucleoside. In order to improve the antitumor activity of 5-fluorouracil, another lipophilic prodrug of 5-fluorouracil with a cholesterol moiety—the cholesteryl 5-(5-fluorouracilcarbamoyl)capronate—was also synthesized as a compound having adequate physicochemical properties for the incorporation in lipidic carriers.[25]

Scheme II



All these attractive research studies prompted us to combine the ideas mentioned above with our purpose of making 7β-hydroxycholesterol water-soluble. Therefore, we have synthesized the conjugate phosphodiester of 7β-OHC and 5F-dUrd or dUrd. In this paper, we describe the preparation of the phosphodiesters [the sodium salt of 5′-(5-fluoro-2′-deoxyuridylyl) 3-(7β-hydroxycholesteryl) phosphate (**1a**) and the sodium salt of 5′-(2′-deoxyuridylyl) 3-(7β-hydroxycholesteryl) phosphate (**1b**) (Scheme I)] and report a preliminary evaluation of their antiproliferative effect in vitro toward EL-4 leukemia cells and their antitumor activity in vivo against the mice bearing Krebs II ascitic carcinoma.

Results

Chemistry. 7β-Hydroxycholesteryl acetate, obtained according to the described method,[10] was converted into 7β-[(triethylsilyl)oxy]cholesteryl acetate in order to protect the 7β-OH group. A selective hydrolysis of the 3-acetate group by 1 N NaOH gave 7β-[(triethylsilyl)oxy]cholesterol (**2**) which was used for phosphorylation (Scheme II).

The bifunctional phosphorylating reagents **4** and **5**, derived from 2-chlorophenyl phosphorodichloridate (**3**) (Scheme III), are widely used for the formation of 3′-5′-internucleotide phosphotriester linkages[26,27] and for the synthesis of complex phosphorus-containing compounds.[28-30] Initially, we explored the feasibility of the synthesis with these two phosphorylating reagents. Sterol **2** was first phosphorylated with **4**, prepared in situ to give **6b** (pathway B), and then coupled directly with nucleoside **8a** or **8b** (pathway D). Unfortunately, the first step gave desired intermediate **6b** only in a very low yield together with a symmetrical phosphotriester of sterol **6′**. Although the formation of this side product could be minimized by using the reagent **5**, which gave intermediate **6c** (pathway C) in high yield (>70%), the second step involving coupling with a nucleoside failed to give a satisfactory yield (pathway D). Therefore, we have tried to carry out the coupling reaction directly with reagent **3** without any activation (pathway A), even though it is not a common method for phosphorylation.

Sterol **2** was treated with 1 equiv of reagent **3** in the presence of a slight excess of triethylamine at 0 °C in THF.

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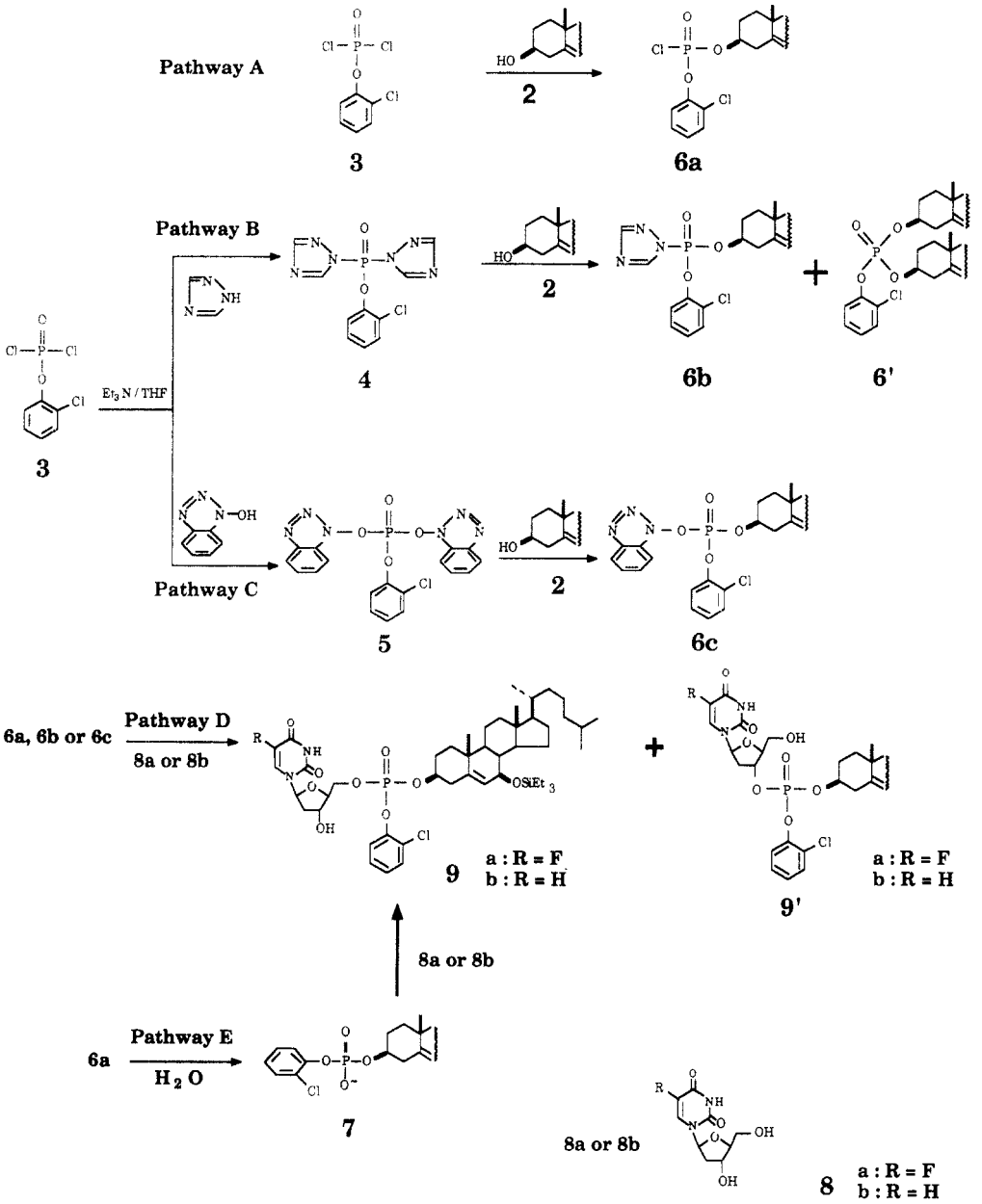
Scheme III



Intermediate **6a**, which was not purified, was allowed to react directly with the 5′-OH group of unprotected nucleoside **8a** or **8b** in the presence of a large excess of DMAP [4-(dimethylamino)pyridine] and afforded triester **9a** or **9b** (pathway D). The intermediate **6a** could also be hydrolyzed to give diester **7**, which could then be coupled with nucleoside **8a** or **8b**, using MSNT [1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole] as condensation agent (pathway E).

In the second step of the direct coupling reaction, the steric hindrance introduced by the sterol nucleus and by the 2-chlorophenyl group at the two P–O bonds led compounds **6a** and **7** to react selectively with the 5′-OH group rather than with the 3′-OH group of nucleoside **8a** or **8b**. In fact, the desired compound **9a** or **9b** was obtained in yields of 30–40%, and the product **9′a** or **9′b**, corresponding to the coupling of the 3′-OH group of nucleoside **8a** or **8b** with compound **6a** or **7**, was produced only in a small amount (2.4%) (Scheme III, pathways D and E).

Purified triester **9a** or **9b** was then deprotected as follows: the triethylsilyl group was removed under weakly acidic conditions and the remaining 2-chlorophenyl group was removed by treatment with an excess of the oximate salt,[31] prepared in situ. The deprotection leading to the desired phosphodiesters **1a** and **1b** was nearly quantitative and the relatively low yield (40%) was due to the sensitivity of the final compounds in their ionic form to purification by chromatography on silica gel.

Biological Results. The antiproliferative activity of the conjugated compounds **1a** and **1b** was first tested in vitro against EL-4 and RDM-4 leukemia cell lines. As shown in the Figure 1, the parent oxysterol 7β-OHC at 20 μM induced the lysis of nearly 95% of the EL-4 cells, after 48 h of culture. **1b** appeared to be less active than 7β-OHC. Moreover, **1a** was less cytotoxic than 5F-dUrd (active at 2.5 μM) but excerts similar antitumor activity to that of 7β-OHC. Thus, these two phosphodiester derivatives of oxysterols seem to be less effective than their parent compounds in vitro. This reduced cytotoxicity may be attributed to failure to be completely hydrolyzed. However, the relatively high activity of **1a**, compared to that of **1b**, indicated the potentiality of the conjugation of antimetabolite nucleosides with cytotoxic oxysterols.

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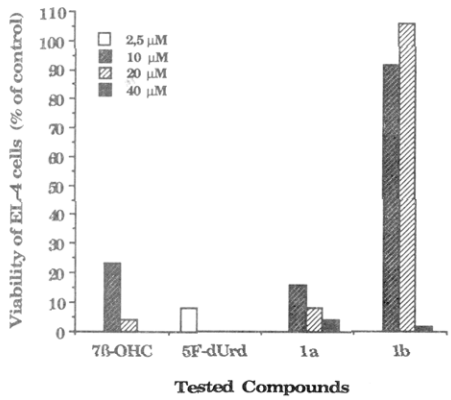

Figure 1. Viability of EL-4 cells, cultured in 10% FCS (fetal calf serum) supplemented medium and treated with various concentrations of 7β-OHC, 5F-dUrd, **1a**, or **1b** for 48 h. Cell viabilities are determined by the Trypan Blue exclusion test and represent percent of control.

Similar results were observed with RDM-4 cells.

Furthermore, a previous study has demonstrated that the cytotoxic activity of the conjugated compounds of type 1 is faster than that of either parent compound.[32] Indeed, in a culture medium free of serum, 2 h of incubation was sufficient to induce a total lysis of 90% of the cells treated by **1a** or **1b**. Under the same conditions, at least 12 h was required for 7β-OHC and 5F-dUrd to express their activity. This rapid cell lysis may be due to the amphiphilic nature of compounds **1a** and **1b**, which could induce a simple detergentlike effect.

As indicated in the introduction, compounds **1a** and **1b** are designed to be used in vivo. The antitumor activity of the conjugate compounds **1a** and **1b** was therefore screened against murine ascitic carcinoma Krebs II and compared with that of their parent compounds—7β-OHC and 5F-dUrd—and with another water-soluble derivative of oxysterol—BHS-7β-OHC. The results are summarized in Table I.

The carcinoma Krebs II cells (1×10^6) were inoculated into OF1 mice and the treatment with the different compounds was initiated 24 h later. Under these conditions, the 5F-dUrd was only moderately active and led to an increase in life span (ILS) of 93%, after the treatment with a dose of 80 μmol/kg per day for 2 consecutive days. The lipophilic oxysterol 7β-OHC failed to inhibit the growth of Krebs II tumor significantly and it gave a ILS of 40%. On the other hand, a treatment with BHS-7β-OHC, at its optimal dose of 84 μmol/kg per day for 3 successive days, gave a remarkable ILS value of 180%, in addition to 30% complete recovery (a survival of more than 80 days). However, the results obtained with conjugated compounds **1a** and **1b** were clearly superior. The treatment with **1a** or **1b** at a dose of 80 μmol/kg per day for 2 days gave a complete recovery rate higher than 90%. The combined administration of the same dose of 7β-OHC and 5F-dUrd (80 μmol/kg per day) did not produce a significant synergic antitumor effect. The addition of dUrd was also unable to modify the antitumor activity of 7β-OHC.

Under the conditions described above, compound **1a** appeared to be as effective as **1b**. Therefore, their antitumor activity seemed to be contributed by the parent oxysterol, as is the case with another water-soluble derivative, BHS-7β-OHC. The high potency of **1a** and **1b** could result from an increase of water solubility of these highly lipophilic molecules, which gives to the desired compounds the appropriate physicochemical properties and which allows them to diffuse quickly in the organism.

Table I. Antitumor Activity in Mice against Intraperitoneally Inoculated Ascitic Krebs II Carcinoma[a]

compd	treat. schedule[b]	dose, μmol/kg per day	median survival time, days	% ILS[c]	% CR[d]
control	–	–	15	–	–
5F-dUrd	1–2	80	29	93	–
7β-OHC	1–2	80	21	40	–
7B-OHC + 5F-dUrd	1–2	80 80	21	40	–
7β-OHC + dUrd	1–2	80 80	21	40	
BHS-7β-OHC	1–3	84	42	180	30
1a	1–2	80	>80	–	>90
	3	80	19	30	20~30
1b	1–2	80	>80	–	>90
	3	80	16	10	–

[a] Female Swiss/OF1 mice in groups of six (average weight of 20–25g) were inoculated ip with 10^6 cells of Krebs II murine carcinoma. [b] Daily treatment (ip) was initiated 1 day or 3 days after tumor inoculation, as indicated in the table. 1–3 indicates three treatments on day 1, 2, and 3; 1–2 indicates two treatments on day 1 and 2. [c] The percent of increase in life span: $[(T/C - 1)] \times 100\%$. [d] CR: complete recovery (percent of animals surviving for more than 80 days).

From the chemotherapeutic point of view, the coupling of 5F-dUrd to 7β-OHC via a phosphodiester bond could render compound **1a** more active than **1b**. In fact, with a delayed treatment starting at day 3 following the inoculation, **1a** was more effective than **1b** in extending the life span of mice bearing Krebs II tumor. After administration of a single dose of **1a** or **1b** of 80 μmol/kg per day at day 3 following tumor transplantation, the former resulted in an ILS of 30%, in addition to the 20–30% of the mice which showed complete recovery. On the other hand, the latter was practically unable to inhibit the growth of tumor.

Discussion

Our main aim in this study was to synthesize the water-soluble derivatives of 7β-hydroxycholesterol, which would be expected to give improved preparations for parental administration for in vivo biological studies. The results presented in this report provide an unambiguous demonstration of the success of our strategy up to now. Indeed, these phosphodiesters **1a** and **1b** have a high water solubility (>30 g/100 mL) and an acceptable degree of hydrolytic stability; for example, they are stable in 1 N NaOH solution for more than 10 days and for several months in a buffer solution at pH ~7. They are also soluble in some organic solvents such as ethanol.

We have undertaken the synthesis of **1a** by coupling two biological compounds which are different in their structural features and their mechanisms of action. Such conjugated compounds possess amphiphilic properties and may counteract some of the disadvantages of either parent compound. In particular, they may overcome the resistance of some tumors to 5F-dUrd.

The mechanism of release of the parent compounds have so far not been evidenced; however, by analogy with similar compounds, in particular with corticosteroids linked to nucleosides by a phosphate bond,[33] it is highly probable that **1a** and **1b** would release their parent compounds by enzymatic hydrolysis. Further evaluation of the effect of these conjugates against other tumor systems known to be

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resistant to 5F-dUrd as well as the mechanism of release of the parent compounds and the study of their metabolism in vivo and in vitro need to be carried out.

Experimental Section

Synthesis. Pyridine and tetrahydrofuran (THF) were dried by refluxing with CaH_2 and $LiAlH_4$, respectively, for several hours, followed by distillation. DMF was distilled under reduced pressure and stored over 4A molecular sieves. All commercial reagents were purchased from Aldrich or Fluka. TLC was run on plates precoated with silica gel (60F 254, Merck), and silica gel (40–63 μm, Merck) was used for column chromatography. Dowex-50 × 8 resin (20–50 mesh, sodium salt) was used for ion-exchange chromatography. Evaporation was performed under reduced pressure at 30 °C. Melting points were measured on a Reichert hot-stage microscope and are uncorrected. $[\alpha]^{20}$ measurements were made with a Perkin-Elmer 141 polarimeter. Microanalyses were performed by the Service Central de Microanalyses of CNRS. NMR spectra were run on a Bruker SY (200- or 400-MHz) apparatus with tetramethylsilane and $CHCl_3$ or CH_3OH as internal standards for ^1H NMR and for ^{13}C NMR, respectively. Mass spectra were recorded on an LKB 9000S apparatus by direct introduction. FAB-MS spectra were recorded by means of a VG analytical ZAB-HF double-focusing mass spectrometer.

7β-[(Triethylsilyl)oxy]cholesterol (2). 7β-Hydroxycholesteryl acetate (444 mg, 1 mmol) was dissolved in dry DMF (5 mL). Diisopropylethylamine (348 μL, 2 mmol) and triethylsilyl chloride (252 μL, 1.5 mmol) were added, and the mixture was kept at room temperature under anhydrous conditions for 4 h. The reaction was quenched by addition of $NaHCO_3$ solution (10%, 2 mL) and extracted with ether (3 × 25 mL). The organic phase was washed with water and evaporated in vacuo.

The crude product was then dissolved in THF (10 mL), and 1 N NaOH (5 mL) and tetrabutylammonium bromide (100 mg) were added. This hydrolysis mixture was vigorously stirred for 3 days at room temperature. The reaction mixture was diluted with ether (100 mL) and washed with brine until the pH was stabilized at 7–8. The organic phase was dried over $MgSO_4$ and concentrated. The oily residue was purified on a short column of silica gel with ethyl acetate and hexane (1/4 v/v) as eluent. Compound **2** (R_f 0.33) was obtained in 98% yield (506 mg): Mp 113.5–114.5 °C; $[\alpha]^{20} = +30°$ (0.66% in $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.68 (s, 3 H, CH_3-18), 0.57–0.68 (m, 6 H, $Si(CH_2CH_3)_3$), 0.87 (d, 6 H, J = 6.6 Hz, CH_3-26,27), 1.05 (s, 3 H, CH_3-19), 0.90–0.99 (m, 12 H: 9 H, $Si(CH_2CH_3)_3$; 3 H, CH_3-21), 3.55 (m, 1 H, H-3), 3.94 (d, 1 H, J = 8 Hz, H-7), 5.26 (s, 1 H, H-6); ^{13}C NMR ($CDCl_3$) see Table II; MS (EI, 70 eV) m/e 516 (M^+, 37), 384 (97), 366 (38). Anal. ($C_{33}H_{60}O_2Si$) C, H, N, Si.

Sodium 3-[7β-(Triethylsiloxy)cholesteryl] 2-Chlorophenyl Phosphate (7). Reagent **3** (164 μL, 1 mmol) and triethylamine (174 μL, 1.25 mmol) were added to a THF (10 mL) solution containing sterol **2** (516 mg, 1 mmol) and the mixture was stirred at 0 °C for 4 h. The workup was performed by addition of a $NaHCO_3$ solution (20%, 4 mL), followed by continuous stirring for 10 min. The reaction mixture was extracted with AcOEt (3 × 25 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo. The residue was purified on a short column of silica gel with CH_3OH/CH_2Cl_2 (3/97 → 10/90) as eluent. The homogeneous product **7** (685 mg) was isolated from the appropriate fractions (yield 94%): R_f 0.35 ($CH_3Cl/CH_3OH/H_2O$ 65/25/4); mp 144–146 °C; ^1H NMR ($CDCl_3$ + CD_3OD) δ 0.59 (s, 3 H, CH_3-18), 0.48–0.59 (q, 6 H, $Si(CH_2CH_3)_3$), 0.78 (d, 6 H, J = 6.5 Hz, CH_3-26,27), 0.82–0.91 (m, 12 H: 9 H, $Si(CH_2CH_3)_3$; 3 H, CH_3-21), 1.03 (s, 3 H, CH_3-19), 3.82 (d, 1 H, J = 8.05, H-7), 4.04 (m, 1 H, H-3), 5.11 (s, 1 H, H-6), 6.91–7.53 (m, 4 H, 2-ClC_6H_4). Anal. ($C_{39}H_{63}O_5ClPSiNa\cdot 2H_2O$) C, H, Cl.

2-Chlorophenyl 5′-(5-Fluoro-2′-deoxyuridylyl)-3-[7β-(Triethylsiloxy)cholesteryl] Phosphotriester (9a). Pathway D. 2-Chlorophenyl phosphorodichloridate (**3**; 164 μL, 1 mmol) was added to a stirred solution of protected sterol **2** (516 mg, 1 mmol) and triethylamine (174 mL, 1 mmol) in dry THF (8 mL) at 0 °C, under argon. The mixture was stirred for 3 h and allowed to warm to room temperature. After 2 h, the reaction mixture was filtered under anhydrous conditions to remove triethylamine hydrochloride. The solution thus obtained was added to pyridine (10 mL) containing 5-fluoro-2′-deoxyuridine (**8a**; 228 mg, 1 mmol), which had previously been dried by coevaporation with pyridine (3 × 25 mL) in vacuo. 4-(Dimethylamino)pyridine (305 mg, 2.5 mmol), dried by the same method, was added to the mixture. After 12 h, workup was carried out by addition of 10% $NaHCO_3$ solution (2 mL) and extraction with AcOEt (3 × 25 mL). The organic phase was dried over $MgSO_4$ and concentrated. The residue was then purified on a short column of silica gel (40–63 μm) with CH_2Cl_2/CH_3OH (97/3) as eluent. Phosphotriester **9a** (373 mg) was obtained as a mixture of diastereoisomers in an overall yield of 40%.

Pathway E. Compound **7** (729 mg, 1 mmol) and 5F-dUrd (**8a**; 228 mg, 1 mmol) were dried by repeatly coevaporation with anhydrous pyridine (3 × 25 mL) and dissolved in pyridine (10 mL). 1-(2-Mesitylenesulfonyl)-3-nitro-1,2,4-triazole (1.48 g, 5 mmol) was added and the mixture was stirred for 24 h. The solution was worked up by addition of 10% $NaHCO_3$ solution (2 mL) and then extraction with CH_2Cl_2 (3 × 25 mL). The organic phase was evaporated and the residue was dissolved in CH_2Cl_2/CH_3OH (97/3). The solution was applied to a short column of silica gel and eluted with CH_2Cl_2/CH_3OH (97/3 → 90/10). The appropriate fractions with an R_f of 0.3 (CH_2Cl_2/CH_3OH 97/3) gave pure triester **9a** (300 mg, 32% yield): ^1H NMR ($CDCl_3$) δ 0.56–0.67 (m, 6 H, $Si(CH_2CH_3)_3$), 0.67 (s, 3 H, CH_3-18), 0.86 (d, 6 H, J = 6.6 Hz, CH_3-26,27), 0.90–0.96 (m, 12 H: 9 H, $Si(CH_2CH_3)_3$; 3 H, CH_3-21), 1.04 (s, 3 H, CH_3-19), 3.91 (br, 1 H, H-7), 4.15 (m, 1 H, H-4′ of 5F-dUrd), 4.43 (m, 3 H, 2 H: H-5′ of 5F-dUrd and 1 H, H-3), 4.51 (m, 1 H, H-3′ of 5F-dUrd), 5.30 (br, 1 H, H-6), 6.3 (t, 1 H, J = 6.4 Hz, H-1′ of 5F-dUrd), 7.15–7.47 (m, 4 H, 2-ClC_6H_4), 7.61–7.71 (d, 1 H, J = 6.3 Hz, H-6 of 5F-dUrd); ^{13}C NMR ($CDCl_3$) see Table II.

The minor compound with an R_f of 0.41 (CH_2Cl_2/CH_3OH 97/3) (20 mg, yield 2.4%) was shown to be phosphotriester **9′a**, resulting from the coupling of 7β-(triethylsiloxy)cholesterol and 3′-OH group of 5F-dUrd: ^1H NMR ($CDCl_3$) δ 0.67 (s, 3 H, CH_3-18), 0.56–0.67 (m, 6 H, $Si(CH_2CH_3)_3$), 0.87 (d, 6 H, J = 6.6 Hz, CH_3-26,27), 0.92–1.00 (m, 12 H: 9 H, $Si(CH_2CH_3)_3$; 3 H, CH_3-21), 1.06 (s, 3 H, CH_3-19), 3.91 (d, 1 H, J = 8.0 Hz, H-7), 3.98 (m, 2 H, H-5′ of 5F-dUrd), 4.26 (m, 1 H, H-4′ of 5F-dUrd), 4.42 (m, 1 H, H-3), 5.24 (m, 1 H, H-3′ of 5F-dUrd), 5.27 (br, 1 H, H-6), 6.31 (t, 1 H, J = 6.4 Hz, H-1′ of 5F-dUrd), 7.13–7.45 (m, 4 H, 2-ClC_6H_4), 8.0 (d, 1 H, J = 6.3 Hz, H-6 of 5F-dUrd).

Sodium Salt of 5′-(5-Fluoro-2′-deoxyuridylyl) 3-(7β-Hydroxycholesteryl) Monophosphate (1a). Compound **9a** (373 mg, 0.4 mmol) was dissolved in a 0.18% HCl solution in THF (15 mL). This mixture was allowed to react for 30 min. A 10% $NaHCO_3$ solution (5 mL) was added and the mixture was then extracted with CH_2Cl_2 (3 × 50 mL). The organic phase was dried over $MgSO_4$ and evaporated in vacuo. The residue was further dried under reduced pressure. The triethylsilyl group was thus removed in 95% yield (311 mg): ^1H NMR ($CDCl_3$) δ 0.67 (s, 3 H, CH_3-18), 0.87 (d, 6 H, J = 6.5 Hz, CH_3-26,27), 0.95 (d, 3 H, J = 6.47 Hz, CH_3-21), 1.05 (s, 3 H, CH_3-19), 3.82 (br, 1 H, H-7), 4.14 (m, 1 H, H-4′ of 5F-dUrd), 4.35–4.62 (m, 4 H: 1 H, H-3; 1 H, H-3′; and 2 H, H-5′ of 5F-dUrd), 5.33 (br, 1 H, H-6), 6.27 (t, 1 H, J = 6.4 Hz, H-1′ of 5F-dUrd), 7.15–7.46 (m, 4 H, 2-ClC_6H_4), 7.67 (d, 1 H, J = 6.5 Hz, H-6 of 5F-dUrd).

The partially deprotected phosphotriester was then treated with 1,1,3,3-tetramethylguanidine (575.9 mg) and 4-nitrobenzaldoxime (1.2 g) in dry THF (5 mL). The mixture was kept at 20 °C until TLC analysis (CH_2Cl_2/CH_3OH 9/1) showed the deprotection to be complete. The solution was then evaporated and the residue was dried under vacuum.

This completely deprotected phosphodiester was purified on a short column of silica gel with CH_2Cl_2/CH_3OH (97/3 → 60/40) as eluent. The appropriate fractions were concentrated and dissolved in water (2 mL). The product was then passed through a column of Dowex-50 × 8 cation-exchange resin (20–50 mesh, sodium salt). The appropriate fractions were lyophilized to give compound **1a** as a white powder: yield 40% (116 mg); ^1H NMR (CD_3OD) δ 0.75 (s, 3 H, CH_3-18), 0.91 (d, 6 H, J = 6.5 Hz, CH_3-26,27), 0.98 (d, 3 H, J = 6.4 Hz, CH_3-21), 1.10 (s, 3 H, CH_3-19), 3.76 (d, 1 H, J = 8.1 Hz, H-7), 4.08 (m, 4 H: 1 H, H-3; 1 H, H-4′; and 2 H, H-5′ of 5F-dUrd), 4.50 (m, 1 H, H-3′ of 5F-dUrd), 5.29 (s, 1 H, H-6), 6.33 (t, 1 H, J = 6.4 Hz, H-1′ of 5F-dUrd), 8.07 (d, 1 H, J = 6.5 Hz, H-6 of 5F-dUrd); ^{13}C NMR (CD_3OD+$CDCl_3$) see Table II; FAB-MS negative (matrix, 1-thioglycerol) 709.0 [(M

Table II. Data Analysis of ^{13}C NMR Spectra[a]

carbon	compound no. (solvent) 7β-OHC ($CDCl_3$)	2 ($CDCl_3$)	5F-dUrd (CD_3OD)	dUrd (CD_3OD)	9a ($CDCl_3$)	1a ($CDCl_3/CD_3OD$)	9b ($CDCl_3$)	1b ($CDCl_3/CD_3OD$)
				Steroid				
1	36.91	38.57			36.54	37.90	36.48	38.57
2	31.54	32.57			29.56	30.95	29.51	31.68
3	71.38	72.34			80.32 d	77.12 d	80.20 d	77.13 d
4	41.69	42.86			41.15	41.12	40.32	41.74
5	143.43	144.10			140.15	143.57	139.98	144.20
6	125.42	127.41			128.02*	127.58	127.96*	128.43
7	73.30	76.47			74.53	73.89	74.77	74.24
8	40.85	41.82			40.28	41.28	40.20	41.70
9	48.23	49.75			47.85	49.54	47.76	49.79
10	36.40	37.90			36.22	37.48	36.17	38.06
11	21.04	22.54			20.96	22.26	20.90	22.83
12	39.52	41.01			39.42	40.72	39.24**	41.22*
13	42.90	44.44			42.91	44.03	42.81	44.58
14	55.43	56.94			55.31***	56.70	55.87***	57.37
15	26.33	28.08			26.53	27.38	26.49	27.82
16	28.47	30.00			28.47	29.69	28.40	30.14
17	55.91	57.88			55.97***	57.27	55.21***	58.03
18	11.77	13.28			11.85	12.90	11.81	13.18
19	19.09	20.23			18.76	19.97	18.76	20.24
20	35.68	37.24			35.76	37.90	35.59	37.56
21	18.72	20.21			18.76	19.86	18.76	20.13
22	36.17	37.68			36.12	37.36	36.05	37.19
23	23.79	25.30			23.76	25.00	23.70	25.48
24	39.45	40.97			39.42	40.93	39.07**	41.41*
25	27.96	29.41			27.92	29.16	27.86	29.87
26	22.49	23.89			22.48	23.59	22.46	23.77
27	22.73	24.11			22.73	23.85	22.71	24.02
				Nucleoside				
2			151.03	153.78	148.89	150.56	150.38	150.41
4			159.90[a]	166.42	156.76[a]	159.31[a]	163.17	163.45
5			142.03[b]	104.23	140.60[b]	141.73[b]	102.71	102.76
6			126.44[c]	144.13	124.08[c]	126.09[c]	139.56	140.17
1′			86.99	87.49	84.93	86.60	84.88	85.54
2′			42.05	40.06	39.42	40.67	39.48**	39.55
3′			72.15	72.42	70.93	71.54	70.90	70.67
4′			89.04	88.70	85.32	87.23	85.04	84.69
5′			62.83	63.13	67.51 d	65.88 d	67.66 d	67.20 d
				Others				
2-ClC_6H_4								
1					146.15		146.68	
2					140.15		139.98	
3					130.71		130.67	
4					126.30*		126.25*	
5					128.49*		128.43*	
6					121.29		121.20	
$Si(CH_2CH_3)_3$		8.41			5.84		5.76	
$Si(CH_2CH_3)_3$		7.33			7.07		7.06	

[a] *, **, ***: interchangeable assignments. a, b, c: doublet. ^{13}C–^{19}F coupling constants (Hz) are as follows:

		5F-dUrd	9a	1a
a	$J_{C_4C_5F}$	25.6	26.3	26.0
b	J_{C_5F}	234.2	238.4	234.9
c	$J_{C_6C_5F}$	34.6	32.9	34.2

d: doublet.

– Na)⁻, 19], 481.0 [(M – Na^+ – Nuc + H^+)⁻, 6], 325.0 [(M – Na^+ – St + H^+)⁻, 13], 129.0 [base, 100]; FAB-MS positive (matrix, TEA) 733 [(M + H^+)⁺, 5], 691 [(M – 18 – Na^+ + H^+)⁺, 8], 367 [(St – 18)⁺, 100]. Anal. ($C_{36}H_{55}N_2O_9FPNa\cdot 2H_2O$) C, H, N, F, P, Na.

2-Chlorophenyl 5′-(2′-Deoxyuridylyl) 3-[7β-(Triethylsiloxy)cholesteryl] Phosphotriester (9b). Compound **9b** was prepared in a yield of 38% in an analogous manner to that for **9a**: ^1H NMR ($CDCl_3$) δ 0.56–0.67 (m, 6 H, $Si(CH_2CH_3)_3$), 0.67 (s, 3 H, CH_3-18), 0.86 (d, 6 H, J = 6.6 Hz, CH_3-26,27), 0.90–0.96 (m, 12 H: 9 H, $Si(CH_2CH_3)_3$; 3 H, CH_3-21), 1.05 (s, 3 H, CH_3-19), 3.92 (br, 1 H, H-7), 4.12–4.15 (m, 1 H, H-4′ of dUrd), 4.41–4.50 (m, 3 H: 2 H, H-5′ of dUrd; 1 H, H-3), 4.53 (m, 1 H, H-3′ of dUrd), 5.31 (s, 1 H, H-6), 5.67 (d, 1 H, J = 8.6 Hz, H-5 of dUrd), 6.32 (t, 1 H, J = 6.2 Hz, H-1′ of dUrd), 7.13–7.50 (m, 4 H, 2-ClC_6H_4), 7.56 (d, 1 H, J = 8.6 Hz, H-6 of dUrd); ^{13}C NMR ($CDCl_3$) see Table II.

The minor compound **9′b** [2-Chlorophenyl 3′-(2′-deoxyuridylyl) 3-[7β-(triethylsiloxy)cholesteryl] phosphotriester] was also identified: ^1H NMR ($CDCl_3$) δ 0.57–0.63 (m, 6 H, $Si(CH_2CH_3)_3$), 0.67 (s, 3 H, CH_3-18), 0.87 (d, 6 H, J = 6.6 Hz, CH_3-26,27), 0.89–1.00 (m, 12 H: 9 H, $Si(CH_2CH_3)_3$; 3 H, CH_3-21), 1.06 (s, 3 H, CH_3-19), 3.83 (br, 1 H, H-7), 3.91 (m, 2 H, H-5′ of dUrd), 4.23 (m, 1 H, H-4′ of dUrd), 4.30 (br, 1 H, H-3), 5.24 (m, 1 H, H-3′ of dUrd), 5.30 (br, 1 H, H-6), 5.75 (d, 1 H, J = 8.1 Hz, H-5 of dUrd), 6.23 (t, 1 H, J = 6.6 Hz, H-1′ of dUrd), 7.12–7.50 (m, 4 H, 2-ClC_6H_4), 7.71 (d, 1 H, J = 8.1 Hz, H-6 of dUrd).

Sodium Salt of 5′-(2′-Deoxyuridylyl) 3-(7β-Hydroxycholesteryl) Monophosphate (1b). Compound **9b** was first treated with a solution of 0.18% HCl in THF to remove the triethylsilyl group, which was then followed by treatment with an oximate salt to remove the 2-chlorophenyl phosphorus protecting group as described for the preparation of **1a**. After pu-

rification by a chromatography on silical gel and passage through a column of Dowex-50 × 8 ion-exchange resin (sodium form), compound **1b** was thus obtained in the yield of 42%: ^{1}H NMR (CD_3OD) δ 0.75 (s, 3 H, CH_3-18), 0.91 (d, 6 H, J = 6.6 Hz, 2 CH_3-26,27), 0.98 (d, 3 H, J = 6.5 Hz, CH_3-21), 1.09 (s, 3 H, CH_3-19), 3.77 (d, 1 H, J = 8.4 Hz, H-7), 3.98–4.08 (m, 4 H; 1 H-3, 1 H-4′, 2 H-5′), 4.55 (m, 1 H, H-3′), 5.31 (s, 1 H, H-6), 5.78 (d, 1 H, J = 8.1 Hz, H-5″), 6.34 (t, 1 H, J = 6.8 Hz, H-1′), 8.01 (d, 1 H, J = 8.1 Hz, H-6″); ^{13}C NMR (CD_3OD) see Table II; FAB-MS positive (matrix, 1-thioglycerol) 737 [MNa^+, 5], 715 [MH^+, 5], 383 [19], 367 [33]; FAB-MS negative (matrix, 1-thioglycerol) 691 [$(M - Na^+)^-$, 36], 481 [$(M - Na^+ - Nuc + H^+)^-$, 9], 307 [$(M - Na^+ - St + H^+)^-$, 35]. Anal. ($C_{36}H_{56}N_2O_9PNa\cdot 2H_2O$) C, H, N, P, Na.

Antiproliferative Activity in Vitro. The murine leukemia EL-4 cells were used to assess the cytotoxic profile of the compounds. Cells were maintained in 25 cm^2 tissue-culture flasks (Falcon 3042F) in RPMI-1640 medium supplemented with 10% inactivated fetal calf serum (Gibco, Bio-Cult, Glasgow, Scotland) and gentalin (20 mg/L). All assays were performed in 24-well plates (Costar). To each well were added 2.5 × 10^5 cells in 2 mL and 5 μL of ethanolic solution of tested compounds. In every case the final ethanol concentration was less than 0.25%. Cells were allowed to proliferate for 48 h at 37 °C in a humified atmosphere containing 5% CO_2. At the end of the incubation, the number of viable cells was determined by the Trypan Blue exclusion test.

Antitumor Activity in Vivo. The compounds shown in Table I were screened for in vivo antitumor activity against intraperitoneally transplanted Krebs II ascitic carcinoma in Swiss/OF1 female mice (supplied by Le Centre d'élevage Iffa Credo/France). The intraperitoneal transplantation of 1 × 10^6 ascitic Krebs II cells (in a 0.25 mL suspension of 0.9% NaCl) in OF1 mice (six mice for each group, average weight 20–25 g) was carried out with donor mice bearing 8–10-day-old tumors. Compounds **1a**, **1b**, or 5F-dUrd were dissolved in 0.9% saline. BHS-7β-OHC was dissolved in ultrapurified water. 7β-OHC was suspended in ultrapurified water and stirred with an electric minimixer to give a homogeneous suspension. A 0.2-mL solution was administered ip daily, starting 24 or 96 h after tumor transplantation, as indicated in the Table I. The mice in the control group received the same volume of 0.9% saline. Animals were observed for 80 days. Antitumor activity was evaluated by comparing the mean survival time of the treated animals (T) with that of saline-treated control animals (C): the percentage of increase in life span, % ILS = $\{(T/C) - 1\} \times 100$, and the percent of animals showing complete recovery (survival for more than 80 days) is %CR. The results presented here are derived from four independent experiments.

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Quinolone Antibacterial Agents Substituted at the 7-Position with Spiroamines. Synthesis and Structure–Activity Relationships

Townley P. Culbertson,* Joseph P. Sanchez, Laura Gambino, and Josephine A. Sesnie

Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, Michigan 48105.
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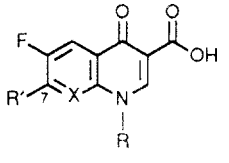
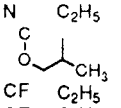
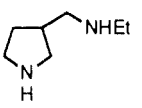
A series of fluoroquinolone antibacterials having the 7-position (10-position of pyridobenzoxazines) substituted with 2,7-diazaspiro[4.4]nonane (**4b**), 1,7-diazaspiro[4.4]nonane (**5a**), or 2,8-diazaspiro[5.5]undecane (**6b**) was prepared, and their biological activities were compared with piperazine and pyrrolidine substituted analogues. Most exhibited potent Gram-positive and Gram-negative activity, especially when side chain **4b** was N-alkylated.

Quinolone antibacterial agents continue to show promise of being an important class of therapeutically useful compounds.[1] Most of these agents, which have broad spectrum activity, are substituted at the 7-position by cyclic aliphatic amines (side chains), especially diamines such as piperazine. Notable examples are norfloxacin (**1a**), ciprofloxacin (**1b**), enoxacin (**1c**), and ofloxacin (**1d**).

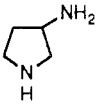


	R′	X	R
1a:	1-piperazinyl	CH	C_2H_5
b:	1-piperazinyl	CH	c-C_3H_5
c:	1-piperazinyl	N	C_2H_5
d:	4-methyl-1-piperazinyl		
e:	3-[(ethylamino)methyl]-1-pyrrolidinyl	CF	C_2H_5
f:	3-amino-1-pyrrolidinyl	CF	C_2H_5
g:	3-[(ethylamino)methyl]-1-pyrrolidinyl	CF	c-C_3H_5
h:	3-amino-1-pyrrolidinyl	CF	c-C_3H_5
i:	5-methyl-2,5-diabicyclo[2.2.1]hept-2-yl	CF	c-C_3H_5

Two other diamines which have been successfully employed are *N*-ethyl-3-pyrrolidinemethanamine (**2**) and 3-aminopyrrolidine (**3**), both of which have been attached to quinolone substrates by way of their ring nitrogen to give the highly active broad spectrum antibacterials **1g** and **1h**, respectively.[2]



2



3

Since flexible linear amines such as ethylenediamine are poor substituents for the 7-position,[1a] it would appear that some rigidity is essential for active quinolone side chains. A recent report on danofloxacin[3] (**1i**) demonstrates that quinolones with side chains less flexible than piperazine can be very active. It was therefore of interest to prepare

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