incubation mixture was rapidly filtered on GF/C filter paper, washed with 10 mL of ice-cold saline, dried, placed in ACS liquid scintillation cocktail, and counted for 5 min each. Data were analyzed using the LIGAND program of Munson and Rodbard,[17] modified for use on an HP 3000 computer. Confidence intervals (95%) were calculated by the method of Munson and Rodbard.[25] K_A values are obtained from pooled data of at least five determinations. Although we used two species for the source of heart tissue (predominantly rat), we observed no species differences in affinities. For *p*-IQNB, $K_A = 1.24 \times 10^9$ M^{-1} (0.79 to 1.9×10^9) for dog, $K_A = 0.77 \times 10^9$ M^{-1} (0.38 to 1.6×10^9) for rat, and $K_A = 1.22 \times 10^9$ M^{-1} (Table I) for the combination of all data.

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Synthesis and Study of a Spin-Labeled Cyclophosphamide Analogue, 3-(1-Oxy-2,2,6,6-tetramethyl-4-piperidinyl)cyclophosphamide[1]

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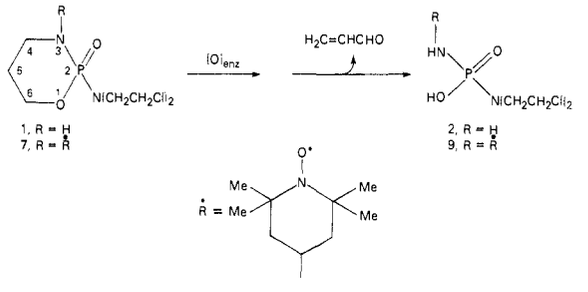
3-(1-Oxy-2,2,6,6-tetramethyl-4-piperidinyl)cyclophosphamide (**7**) was isolated in 36% yield following H_2O_2–Na_2WO_4 oxidation of 3-(2,2,6,6-tetramethyl-4-piperidinyl)cyclophosphamide (**6**), which was synthesized in three steps (25% yield) starting with 4-amino-2,2,6,6-tetramethylpiperidine. Binding of **7** to mouse liver microsomes was investigated by optical and electron spin resonance spectroscopy. Compared with the mouse liver microsomal metabolism of **1**, separate incubations of **6** and an ca. 1:1 mixture of **1** and **6** gave approximately 90 and 60% *less* acrolein, respectively. A spin-labeled metabolite of **7**, viz., *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)phosphoramide mustard (**9**), was synthesized and its intramolecular O-alkylation at pH 7.4, 37 °C, was studied by ^{31}P NMR spectroscopy. Compounds **7** and **9** were inactive in screening tests against L1210 lymphoid leukemia in mice.

While numerous analogues[2] of cyclophosphamide (**1**) have been employed for investigating the mechanism of action of this widely used anticancer prodrug,[3] spin-labeling[4,5] studies dealing with **1** have not been reported. On the other hand, spin-labeled analogues of the antitumor agents thio-TEPA[6-8] and 5-aziridino-2,4-dinitrobenzamide[9] have exhibited oncostatic selectivity;[8-10] moreover, the sterically protected, paramagnetic, 1-oxo-4-piperidinyl moiety in these molecules has been utilized for novel pharmacokinetic measurements by ESR spectroscopy.[9,10]

Scheme I



Two key features of cyclophosphamide metabolism[3] (Scheme I) are the initial "activation" (C-4 oxidation) of **1** by a liver microsomal mixed-function oxidase and the eventual release of the cytotoxic phosphoramide mustard (**2**) fragment. Attachment of a free radical to the endocyclic nitrogen (N-3) in **1** provides a spin-labeled analogue that could be used to probe metabolic and dynamic processes involving **1**, **2**, and intermediary phosphorus-containing metabolites—provided that the analogue undergoes efficient "activation" and that the paramagnetic center is persistent under biological conditions. In view of the success of the 1-oxy-2,2,6,6-tetramethyl-4-piperidinyl group as a spin-label,[6-10] 3-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)cyclophosphamide (**7**) was investigated as a *prototype for appraising the feasibility of the ESR "reporter group" method in studies of cyclophosphamide metabolism.*

Results and Discussion

The strategy for conversion of starting material **3** into 3-(2,2,6,6-tetramethyl-4-piperidinyl)cyclophosphamide (**6**) was similar to that reported[11] for the synthesis of 3-(α-methylbenzyl)cyclophosphamide. A mixture of H_2O_2 and Na_2WO_4 selectively oxidized the nitrogen–hydrogen bond in **6** and, thus, afforded analytically pure **7** after silica gel chromatography. Since the utility of any spin-labeled analogue is dependent upon, inter alia, its ability to mimic the parent molecule, several lines of investigation were

(1) This paper is part 5 of a series on "Synthesis and Antitumor Activity of Cyclophosphamide Analogues". For part 4, see J. A. Brandt, S. M. Ludeman, G. Zon, J. A. Todhunter, W. Egan, and R. Dickerson, *J. Med. Chem.*, **24**, 1404 (1981). Systematic nomenclature is given under Experimental Section.

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(3) O. M. Friedman, A. Myles, and M. Colvin, *Adv. Cancer Chemother.*, 1, 143–204 (1979), and pertinent references therein.

(4) L. J. Berliner in "Spin Labeling Theory and Applications", L. J. Berliner, Ed., Academic Press, New York, 1976, pp 1–4.

(5) G. I. Likhtenshtein, "Spin Labeling Methods in Molecular Biology", Wiley-Interscience, New York, 1976, pp 190–232.

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(7) G. Sosnovsky, Y.-I. Yeh, and G. Karas, *Z. Naturforsch.*, **28c**, 781 (1973).

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(10) N. M. Emanuel, N. P. Konovalova, and R. F. Djachkovskaya, *Cancer Treat. Rep.*, **60**, 1605 (1976).

(11) G. Zon, *Tetrahedron Lett.*, 3139 (1975).

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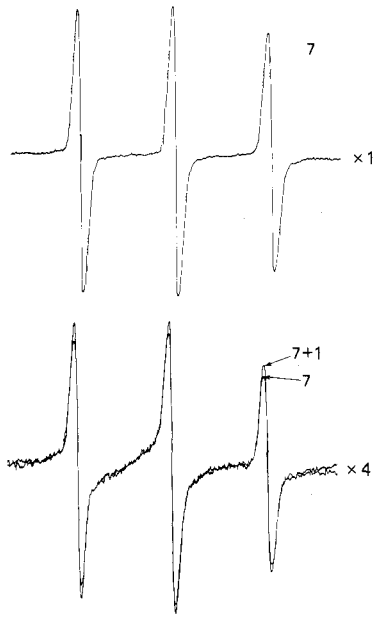


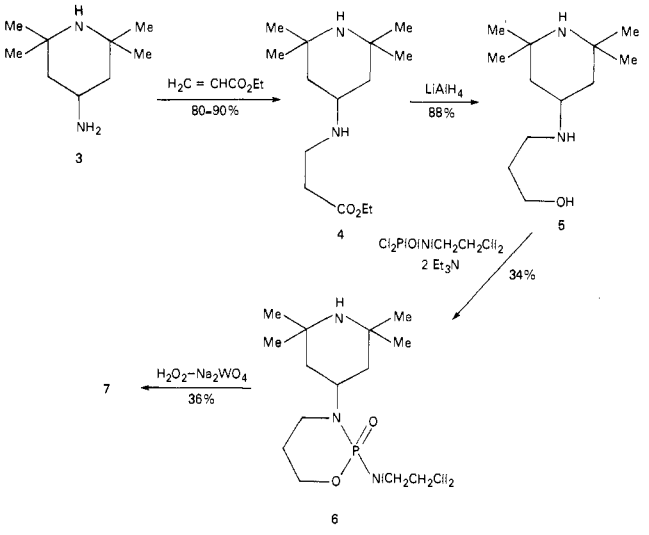
Figure 1. Top: ESR spectrum of **7** (4×10^{-5} M) in phosphate buffer (0.25 M, pH 7.4); $g_0 = 2.01222$ and $A_N = 16.8$ G. Bottom: ESR spectra of **7** (4×10^{-5} M) in phosphate buffer (0.25 M, pH 7.4) containing phenobarbital-induced mouse liver microsomes (1 g of whole liver equiv/mL) before and after addition of **1** (5.4×10^{-4} M). The relative display amplitudes are indicated as ×1 and ×4.

directed at evaluating the influence of the substantial structural differences between analogues **7** and **1**.

Binding of drugs and other chemicals to hepatic cytochrome P-450 affords two classes of difference spectra, which are referred to as types I and II and are characterized by λ_{max} ranges of 385–390 and 425–435 nm, respectively, and equally broad λ_{min} ranges of 418–427 and 390–405 nm, respectively.[12] Sladek[13] has reported that **1** bound with phenobarbital-induced rat liver microsomal cytochrome P-450 elicited a type I spectrum; from the published[13] absorption curve, we estimated a λ_{max} of 394 nm and a λ_{min} of 430 nm. Similar measurements using **7** (0.22 mM) and liver microsomes (2.6 mg protein/mL) obtained from phenobarbital-treated mice gave a λ_{max} of 390 nm, a λ_{min} of 412 nm, and a small ΔA value ($A_{390} - A_{412} \simeq 0.008$), relative to 1.[13] These findings *suggested* that **7** has a slight bias toward type I binding.[14]

ESR spectroscopy was used as a second method for comparing the interactions of **1** and **7** with hepatic microsomal hemoprotein. The triplet ESR pattern (Figure 1, top) for a 4×10^{-5} M solution of **7** in phosphate buffer was virtually identical with the spectrum similarly recorded (not shown) for 4-amino-1-oxy-2,2,6,6-tetramethylpiperidine (**8**) and, thus, was characteristic of a "free" (motionally unrestricted) spin-labeled molecule. The presence of phenobarbital-induced microsomes in an otherwise identical sample of **7** caused several changes in the ESR spectrum (Figure 1, bottom): (1) the peak heights were reduced approximately 4-fold, (2) the relative heights of the triplet components (0.95:1:0.71) were slightly "distorted" by comparison with the values for **7** alone (1:1:0.84), and (3) a broadened base-line component was also visible. These spectral changes were qualitatively similar to those reported[15a] for binding of a 1-oxy-2,2,6,6-tetramethyl-4-piperidinyl analogue of nicotinamide adenine dinucleotide to liver alcohol dehydrogenase. That the spectral alterations *may* be due to selective binding of **7** to the cytochrome P-450 component of the microsomes was supported by control spectra recorded (not shown) for (1) **7** with added protein (bovine serum albumin), (2) **7** with added calf thymus DNA, and (3) **8** with added mouse liver microsomes. In each case there was no discernible difference between spectra recorded before and after addition of the biological materials (cf. Experimental Section). Addition of **1** to the sample containing **7** and the liver microsomes caused an increase in the ESR peak heights; however, the effect was relatively small, i.e., a 13.5 M excess of **1** gave a ca. 10% signal enhancement (Figure 1, bottom). While this observation was *consistent* with competitive displacement of a fraction of bound (immobilized) **7** by the parent drug molecule, nonspecific binding of **7** with, for example, lipid components must also be considered. Unfortunately, more detailed ESR studies of the liver microsomal binding could not be pursued due to the gradual reduction of **7**, presumably by protein sulfhydryl groups.[15b]

Scheme II



The third method for comparing the interactions of **1** and **7** with liver microsomes involved fluorometric monitoring of the acrolein resulting from substrate "activation" in the presence of required cofactors. Under conditions normally used for microsomal oxidative "activation" of **1** and its analogues, 1-oxy-2,2,6,6-tetramethylpiperidine has been enzymatically *reduced* to its corresponding hydroxylamine;[16] consequently, precursor **6** (Scheme II) was used as an isosteric substitute for **7** to obviate possible complications arising from competing redox processes. Figure 2 shows the time courses for acrolein detection during separate incubations of **1**, **6**, and an ca. 1:1 mixture of **1**

(12) G. J. Mannering in "Fundamentals of Drug Metabolism and Drug Disposition", B. N. LaDu, H. G. Mandel, and E. L. Way, Eds., Williams & Wilkins, Baltimore, 1971, pp 219–233.
(13) N. E. Sladek, *Cancer Res.*, **31**, 901 (1971).
(14) Mannering[12] has discussed the possibility that few if any compounds combine exclusively with either the type I or type II binding site and that compounds produce composite spectra which in varying degrees are only predominantly type I or type II. Hence, a compound capable of combining equally well with both binding sites would present essentially no difference spectrum, and the molar extinction coefficient of a type I (or type II) compound would be reduced by whatever degree the compound also combines with the type II (or type I) site.
(15) (a) H. Weiner, *Biochemistry*, **8**, 526 (1969); (b) F. R. Robey, G. A. Jamieson, and J. B. Hunt, *J. Biol. Chem.*, **254**, 1010 (1979); J. D. Morrisett and H. R. Drott, *J. Biol. Chem.*, **244**, 5083 (1969); A. J. Marinello, H. L. Gurtoo, A. J. Marinello, H. L. Gurtoo, R. F. Struck, and B. Paul, *Biochem. Biophys. Res. Commun.*, **83**, 1347 (1978).
(16) G. M. Rosen and E. J. Rauckman, *Biochem. Pharmacol.*, **26**, 675 (1977).

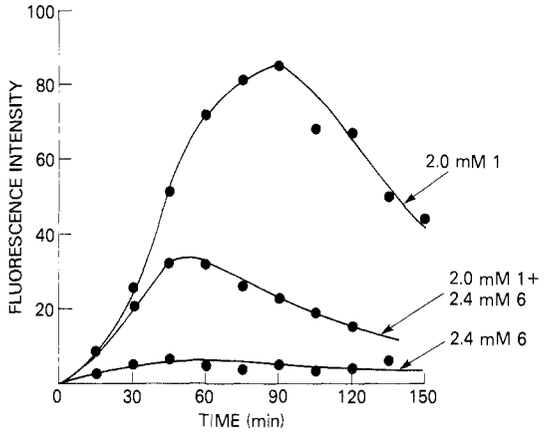


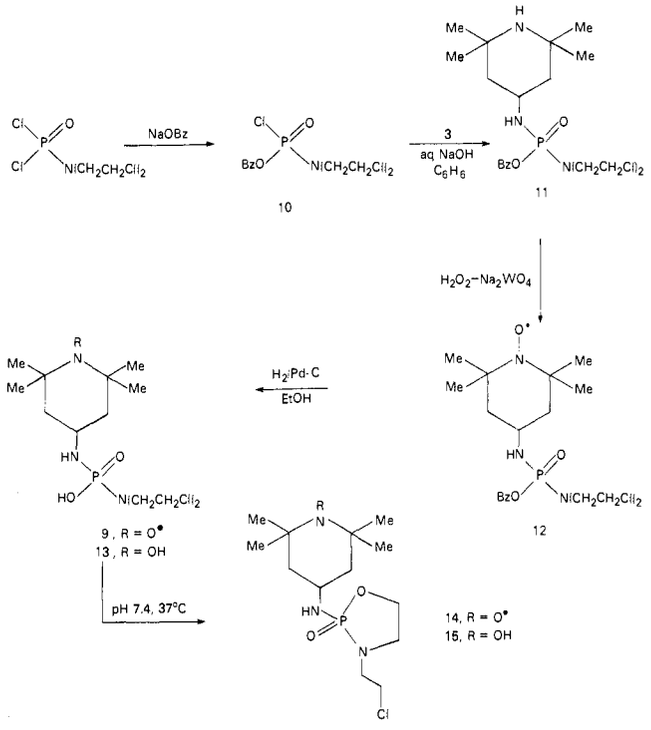
Figure 2. Time courses of acrolein removal from separate incubation (37 °C) mixtures in phosphate buffer (0.25 M, pH 7.4) having the indicated substrate concentrations and containing phenobarbital-induced mouse liver microsomes (0.75 g of whole liver equiv) plus cofactors. In each case the final volume was 10 mL, and the flow rate for O_2 carrier gas was 120 mL/min. The relative concentration of acrolein, which was collected in cold water traps (60 mL, replaced every 15 min), is given as fluorescence intensity.

and **6** using the same preparation of phenobarbital-induced mouse liver microsomes. The production of acrolein by incubation of **6** demonstrated that this model compound underwent enzyme-mediated C-4 oxidation and subsequent fragmentation; however, the total amount of acrolein from **6** was only ca. 10% of that detected starting with 1.[17] This finding *suggested* that the efficiency of the enzymatic oxidation of **6** was substantially less than that of 1, while the coincubation results (ca. 40% of the acrolein produced by 1) provided evidence for competitive (rather than parallel) enzymatic processing of the two substrates, 1 and **6**.

In principle, steric and electronic effects of the spin-label substituent in the expected metabolite from **7** (**9**, Scheme I) must influence the chemistry of **9** relative to phosphoramide mustard (**2**). Scheme III outlines the synthesis of **9**, which was co-isolated with byproduct **13** as a 1:1 mixture of cyclohexylammonium (CHA) salts. The mixture of **9**·CHA and **13**·CHA was, nevertheless, suitable for half-life ($\tau_{1/2}$) measurements by ^{31}P NMR spectroscopy.[18] Two resonance signals (δ 13.02 and 12.67) were resolved at pH 7.4, 37 °C, and the derived lifetimes were *roughly* comparable: $\tau_{1/2}$ = 51 min for δ 13.02, and $\tau_{1/2}$ = 34 min for δ 12.67; hence, signal identification was not pursued. Under identical reaction conditions, **2**·CHA has exhibited a half-life of 18 min.[18,19] In addition to these *kinetic* differences between **9** and **2**, the decomposition *products* from **9**/**13** and **2** were quite dissimilar: the former compounds underwent intramolecular O-alkylation to give **14**/**15** (Scheme III) in 40% yield whereas **2**·CHA afforded the analogous five-membered ring product in <2% yield.[19]

Spin-labeled cyclophosphamide analogue **7** and the 1:1 mixture of **9**/**13**·CHA were compared with cyclophosphamide in standard anticancer-screening tests against L1210

Scheme III



lymphoid leukemia using male BDF_1 mice given an inoculum of 10^5 cells. In contrast to the ca. 64% increased life span caused by ip injections of cyclophosphamide at a dose of 100 mg/kg, neither test sample gave indications of anticancer activity at sublethal doses (75–300 and 25–100 mg/kg, respectively).

Conclusions

The spectroscopic and chemical data obtained with **7** and **6** indicated that attachment of the relatively bulky 2,2,6,6-tetramethyl-4-piperidinyl moiety to the N-3 position of 1 did not preclude "cyclophosphamide-like" liver microsomal binding and C-4 oxidation but significantly diminished the extent of lethal fragmentation that follows enzymatic "activation". In addition, the nitroxyl substitutent in metabolite analogue **9** caused extensive intramolecular formation of **14**, which is a "one-armed" alkylating agent; incursion of this pathway must significantly alter the biological chemistry of **9** by comparison with **2**.[20] In summary, further investigations of the metabolism of 1 using the present spin-labeling approach were unwarranted.

Experimental Section

Palladium (10%) on charcoal powder (Pd/C) was obtained from Pfaltz and Bauer, Inc. Melting points were obtained with a Thomas-Hoover apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Inc. ^1H NMR refers to 60-MHz spectra that were recorded with a Varian EM360-A instrument. ^{31}P FT NMR (40.25 MHz) and ESR spectra were obtained with JEOL FX-100 and Varian Model V-4535 instruments, respectively. Mass spectra were recorded with an LKB Model 2091 GC–MS system using a solids probe inlet and 70 eV for ionization.

Synthesis of *N*,*N*-Bis(2-chloroethyl)-3-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)tetrahydro-2*H*-1,3,2-oxazaphosphorin-2-amine 2-Oxide (7). Ethyl acrylate (6 mL, 55.4 mmol) was added slowly (10 min, N_2 atmosphere) to a solution (45 °C) of **3** (6.01 g, 38.5 mmol) in absolute EtOH (14 mL), and the mixture was then refluxed for 1.5 h. The reaction mixture was stirred at room temperature overnight, and the mixture was then

(17) Acrolein formation by **6** could also result from liver microsomal oxidation of the piperidinyl carbon–hydrogen bond adjacent to N-3, which leads to 1. Such "side-chain oxidation" has precedent in the metabolism of isophosphamide [K. Norpoth, *Cancer Treat. Rep.*, **60**, 437 (1976)].

(18) T. W. Engle, G. Zon, and W. Egan, *J. Med. Chem.*, **22**, 897 (1979).

(19) A detailed report concering the kinetics and chemistry of **2** and other N-substituted phosphoramide mustards will be published elsewhere.

(20) L. C. Erickson, L. M. Ramonas, D. S. Zaharko, and K. W. Kohn, *Cancer Res.*, **40**, 4216 (1980).

concentrated on a rotary evaporator. The resultant oil was Kugelrohr distilled to give **ethyl 3-[(2,2,6,6-tetramethyl-4-piperidinyl)amino]propionate** (**4**; 7.84 g, 30.6 mmol, 80%), bp 125–135 °C (5 mm); ^{1}H NMR ($CDCl_3$–Me_4Si) δ 4.18 (q, J = 7 Hz, 2, OCH_2), 3.27–2.37 (m, 5, CH_2CH_2 and NCH), 1.20 (t, J = 7 Hz, 3, CH_2CH_3), 2.10–0.60 (m, 18, remaining NH, CH_2, and CH_3 groups), NH not observed. A duplicate preparation gave a 90% yield of **4**. A solution of **4** (3.89 g, 15.2 mmol) in THF (30 mL) was added slowly (30 min, N_2 atmosphere) to a mixture of $LiAlH_4$ (1.73 g, 45.6 mmol) and THF (50 mL). The reaction mixture was refluxed for 1 day and was then successively treated (25 °C) with H_2O (1.8 mL), 15% aqueous NaOH (1.8 mL), and more H_2O (5.4 mL). The precipitate was removed and the filtrate was then concentrated to give a residual oil, which was dissolved in $CHCl_3$ (75 mL) and dried with $MgSO_4$. Rotatory evaporation of the $CHCl_3$ gave a solid (2.68 g), which was combined with similar material (0.16 g) obtained by THF extraction (Soxhlet, 16 h) of the aforementioned precipitate. The resultant **3-[(2,2,6,6-tetramethyl-4-piperidinyl)amino]propanol** (**5**; 2.84 g, 13.3 mmol, 88%) was purified by recrystallization: mp 70–73 °C ($CHCl_3$–Et_2O); ^{1}H NMR ($CDCl_3$–Me_4Si) δ 3.80 (t, J = 6 Hz, 2, OCH_2), 3.18–2.61 (m, 3, NCH_2 and NCH), 2.3 (br s, 3, NH and OH), 2.08–0.60 (m, 18, remaining CH_2 and CH_3 groups). Anal. ($C_{12}H_{26}N_2O$) C, H, N. Intermediate **5** (2.14 g, 10 mmol), Et_3N (2.78 mL, 20 mmol), and a solution of *N,N*-bis(2-chloroethyl)-phosphoramidic dichloride (2.59 g, 10 mmol) in EtOAc (20 mL) were sequentially added to EtOAc (20 mL, N_2 atmosphere). The reaction mixture was stirred (2 days, 25 °C) and suction filtered, and the concentrated filtrate was chromatographed on silica gel (60–200 mesh, 1:1 $CHCl_3$–MeOH), which gave ***N,N*-bis(2-chloroethyl)-3-(2,2,6,6-tetramethyl-4-piperidinyl)tetrahydro-2*H*-1,3,2-oxazaphosphorin-2-amine 2-Oxide** (**6**) as an oil (1.34 g, 3.35 mmol, 34%): R_f 0.37 (silica gel, 1:1 $CHCl_3$–MeOH, I_2 visualization); ^{31}P NMR (40.25 MHz, $CDCl_3$) δ 11.98 relative to external 25% aqueous H_3PO_4; ^{1}H NMR ($CDCl_3$–Me_4Si) δ 4.49–3.84 (m, 2, OCH_2), 3.84–2.88 (m, 12, NCH_2CH_2Cl, NCH, NCH_2, and NH), 2.18–0.80 (m, 18, remaining CH_2 and CH_3 groups). A mixture of **6** (0.54 g, 1.35 mmol) and H_2O (10 mL) was stirred vigorously with H_2O_2 (0.36 mL, 30% aqueous solution) and $Na_2WO_4 \cdot 2H_2O$ (10 mg, 0.03 mmol) for 4 days at room temperature. The reaction mixture was saturated with K_2CO_3 and extracted with Et_2O (20 mL, 3 times), and the combined Et_2O layers were dried ($MgSO_4$) and then concentrated to give a residue, which was applied to two preparative TLC plates (silica gel, 2-mm layer, 20 × 20 cm) for elution with 8:2 $CHCl_3$–MeOH. A red-colored band (R_f 0.80) was desorbed with 1:1 $CHCl_3$–MeOH to give a viscous red oil (0.20 g, 0.48 mmol, 36%), which crystallized (mp 93–94 °C) upon standing in vacuo. Anal. ($C_{16}H_{31}N_3O_3PCl_2$) C, H, N. Unreacted **6** (R_f 0.20; 0.034 g, 0.08 mmol, 6%) was recovered from the TLC plates in a similar manner.

Synthesis of *N,N*-Bis(2-chloroethyl)-*N'*-(1-oxy-2,2,6,6-tetramethyl-4-piperdinyl)phosphoradiamidic Acid Cyclohexylammonium Salt (9·CHA). The preparation and subsequent reaction of **benzyl *N,N*-bis(2-chloroethyl)phosphoramidochloridate** (**10**) were modifications of the procedures reported by Friedman et al.[21] To an ice-cooled, magnetically stirred solution of *N,N*-bis(2-chloroethyl)phosphoramidic dichloride (2.58 g, 0.01 mol) in C_6H_6 (25 mL) was added (20 min) a slurry of sodium benzylate (0.01 mol) in C_6H_6 (25 mL). The reaction mixture was stirred for 2 h at ice bath temperature and was then allowed to warm to room temperature before the sequential addition of freshly distilled **3** (1.56 g, 0.01 mol) and 5 N NaOH (50 mL). After several minutes, the reaction flask became warm and it was then cooled with an ice bath for continued stirring (4 h). The organic layer was separated, washed with H_2O (50 mL), dried with $MgSO_4$, and concentrated, and the resultant semicrystalline mass was then purified by "flash" chromatography[22] (230–400 mesh silica gel, *i*-PrOH). **Benzyl *N,N*-bis(2-chloroethyl)-*N'*-(2,2,6,6-tetramethyl-4-piperidinyl)phosphorodiamidate** (11; 1.15 g, 2.6 mol, 26% yield) was thus obtained as an oil; ^{1}H NMR ($CDCl_3$–Me_4Si) δ 7.30 (s, 5, C_6H_5), 5.05 (d, J = 8 Hz, 2, OCH_2), 3.80–3.20 (m, 8, NCH_2CH_2Cl), 2.00 (m, 4), 1.20 (s, 12). Intermediate **11** (203 mg, 0.45 mmol) was suspended in a mixture of H_2O (2.4 mL) and EtOH (0.5 mL), and 30% aqueous H_2O_2 (0.3 mL) was then added followed by $Na_2WO_4 \cdot 2H_2O$ (7.2 mg). After the mixture was stirred for 7 days at room temperature, 0.2 M K_2CO_3 was added until a persistent turbidity was seen, and the mixture was then extracted with Et_2O (3 mL, 2 times). Purification of the concentrated extract by chromatography (60–200 mesh silica gel, CH_2Cl_2) gave **benzyl *N,N*-bis(2-chloroethyl)-*N'*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)phosphorodiamidate** (**12**) as a red-colored solid, mp 79–82 °C. Anal. ($C_{20}H_{33}N_3O_3PCl_2$) H, N, Cl; C: calcd, 51.62; found, 52.48. Hydrogenolysis of **12** (100 mg, 0.22 mmol) in absolute EtOH (5 mL) was carried out at room temperature using 1 atm of H_2 and 10% Pd/C (50 mg). After 1 equiv of H_2 (5 mL) was consumed (30 min), cyclohexylamine (0.02 mL) was added, and the catalyst was then removed by filtration. Concentration of the filtrate gave **9/13**·CHA (51 mg, 50%): ^{31}P NMR (Me_2SO-d_6) δ 18.82 and 18.55, relative to external 25% H_3PO_4, 1:1 signal intensities. A 4 × 10^{-5} M solution of **9/13**·CHA gave an ESR spectrum with the same g_0 and A_N values as those recorded with a 4 × 10^{-5} M solution of 4-hydroxy-1-oxy-2,2,6,6-tetramethylpiperidine; however, the signal intensity for the mixture was 46% of that obtained with the latter reference compound.

Preparation of Mouse Liver Microsomes. Male Dub ICR mice (3–4 weeks old, ~25 g) were pretreated with phenobarbital by ip injections: 30 mg/kg in the first morning and evening and 60 mg/kg in the morning for 2 consecutive days. The animals were deprived of food for 8–10 h before cervical dislocation and isolation of liver microsomes according to a previously reported method.[13] For incubation experiments, the microsomal pellets were pooled and suspended in 0.25 M phosphate buffer (pH 7.4) at 5 °C to give a microsome concentration equivalent to 1 g of whole liver per milliliter of suspension. The cytochrome P-450 content of the liver microsomes was 1.5 nmol of cytochrome P-450/mg of protein, as determined by standard assay methods for cytochrome P-450[23] and protein.[24]

Binding Studies with Mouse Liver Microsomes. The difference spectrum was obtained in a manner similar to that reported[13] for **1**. A microsomal suspension in 1.15% aqueous KCl (2.6 mg of protein/mL) was added to the reference and sample cuvettes for establishing a base line. A solution of **7** in H_2O (50 μL) was added to the sample cuvette (final concentration of **7** = 0.22 mM), and H_2O (50 μL) was added to the reference cuvette. The spectrum was scanned from 350 to 500 nm using an optical density range of −0.05 to +0.05 A.

The following samples were used for ESR measurements (Figure 1) at ambient probe temperature (25 °C): 10 μL of 1.21 × 10^{-3} M **7** in H_2O added to 0.3 mL of 0.25 M phosphate buffer (pH 7.4); 10 μL of 1.21 × 10^{-3} M **7** in H_2O added to 0.3 mL of the microsomal suspension (vide supra); 1 μL of 1.21 × 10^{-2} M **7** in H_2O added to 0.3 mL of the microsomal suspension, followed by 9 μL of 1.21 × 10^{-2} M **1** in H_2O. ESR control spectra using bovine serum albumin (BSA) were recorded with three samples containing 2, 8, and 40 μL of 3.70 × 10^{-4} M **7** in H_2O added to 0.3-mL aliquots of a BSA solution (100 mg in 10 mL of 0.07 M phosphate buffer with 5% NaCl, pH 7.0). ESR control spectra using DNA were recorded with three samples containing 2, 8, and 40 μL of 3.70 × 10^{-4} M **7** in H_2O added to 0.3-mL aliquots of a calf thymus DNA solution (10 mg in 10 mL of 0.07 M phosphate buffer with 5% NaCl, pH 7.0). A control spectrum using **8** was recorded with a sample prepared from 10 μL of 1.21 × 10^{-3} M **8** in H_2O added to 0.3 mL of the microsomal suspension.

Liver Microsome Induced Formation of Acrolein. The procedure reported by Alarcon et al.[25,26] for monitoring the production of acrolein was adapted as previously described;[27] per-

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tinent details are given in the caption for Figure 2.

^{31}P NMR Kinetic Measurements and Identification of 14/15. The procedure described[18] for 2·CHA was applied to a solution of 9/13·CHA (1:1, 30 mg) in 1 M Tris buffer (1.8 mL, pH 7.4) diluted with D_2O (0.2 mL). The two starting material signals (δ 13.02 and 12.67, relative to external 25% H_3PO_4) in nine spectra that were recorded during 1 h of reaction at 37 °C were used to obtain plots of ln (% P) vs. time, where % P = [(starting material signal intensity)/(total signal intensity)] × 100; for δ 13.02, slope = -2.04×10^{-2} min^{-1}, correlation coefficient = 0.987; for δ 12.67, slope = -1.72×10^{-2} min^{-1}, correlation coefficient = 0.999. After 76% reaction (average for 9·CHA and 13·CHA), the combined signal intensity for 14/15 (δ 29.45, 29.05) was 30% of the total signal intensity. The NMR sample was then saturated with NaCl and extracted with $CHCl_3$ (1 mL, 2 times); MS, m/z 338 and 340 for 14 and m/z 339 and 341 for 15.

Anticancer Screening Tests. Male BDF_1 mice (6 weeks old) were given an inoculum of 1×10^5 L1210 cells on day 0, and the test compounds were administered (ip) on day 2 using groups of six to seven mice and either 10% aqueous Me_2SO or corn oil as the vehicle. At a dose of 100 mg/kg, 1 in either vehicle gave an increased life span (ILS) of ~64%, relative to the control groups (14–18 mice), which received ip injections of either vehicle (5 mL/kg) on day 2. Compound 7 in 10% aqueous Me_2SO gave ILS values of 11.0, −0.7, and 3.2% at doses of 75, 150, and 300 mg/kg, respectively; 9/13·CHA (1:1) in corn oil gave ILS values of 4.9, 1.5, and 1.7% at doses of 25, 50, and 100 mg/kg.

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Antiinflammatory Properties of 8-Aryl-5-isopropyl-2*H*-1,3-dioxolo[4,5-*g*]quinazolin-6(5*H*)-ones and -thiones

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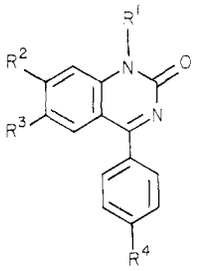
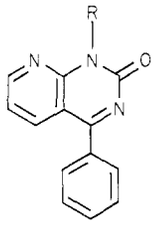
A series of 8-aryl-5-isopropyl-2*H*-1,3-dioxolo[4,5-*g*]quinazolin-6(5*H*)-ones and -thiones was prepared and evaluated for antiinflammatory activity. The 8-phenyl-, 8-(3-fluorophenyl)-, and 8-(4-fluorophenyl)-2*H*-1,3-dioxolo[4,5-*g*]-quinazolin-6(5*H*)-ones and the 8-phenyl-2*H*-1,3-dioxolo[4,5-*g*]quinazolin-6(5*H*)-thione were found to exhibit activity in the range of indomethacin and proquazone.

Since the early 1960's a considerable effort has been expended by medicinal chemists in developing aryl and aralkyl acids for use as nonsteroidal antiinflammatory drugs[1] (NSAIDS). In recent years a number of nonacidic agents, such as indoxole,[2] diftalone,[3] and nictindole,[4] have been found to exhibit an antiinflammatory profile similar to the acidic drugs.[5] In our laboratories a search for nonacidic NSAIDS has led to the findings that 1-alkyl-4-aryl-2(1*H*)-quinazolinones[6,7] (1) possess a good level of

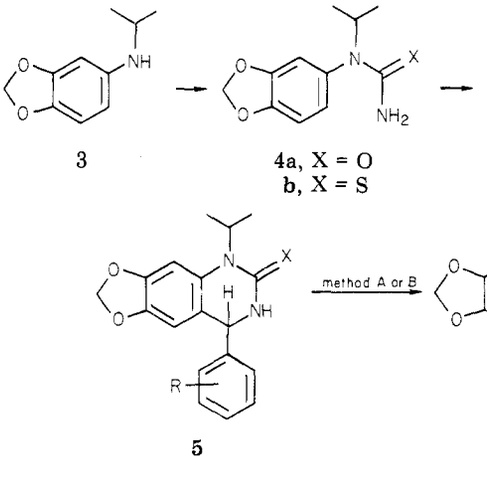




2

	R^1	R^2	R^3	R^4
1a	*i*-C_3H_7	CH_3	H	H
b	*i*-C_3H_7	CH_3	H	F
c	CH_2-c-Pr	H	OCH_3	H

Scheme I



3 4a, X = O; b, X = S

5 6, X = O; 7, X = S

antiinflammatory activity. From this series, the clinically useful proquazone[8] (**1a**) and fluproquazone[9] (**1b**) have been developed for treatment of rheumatoid arthritis and various types of pain, and the related ciproquazone[10] (**1c**) has

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