Synthesis of 3-Hydroxycyclophosphamide and Studies Related to Its Possible Role in the Metabolism of Cyclophosphamide[1]

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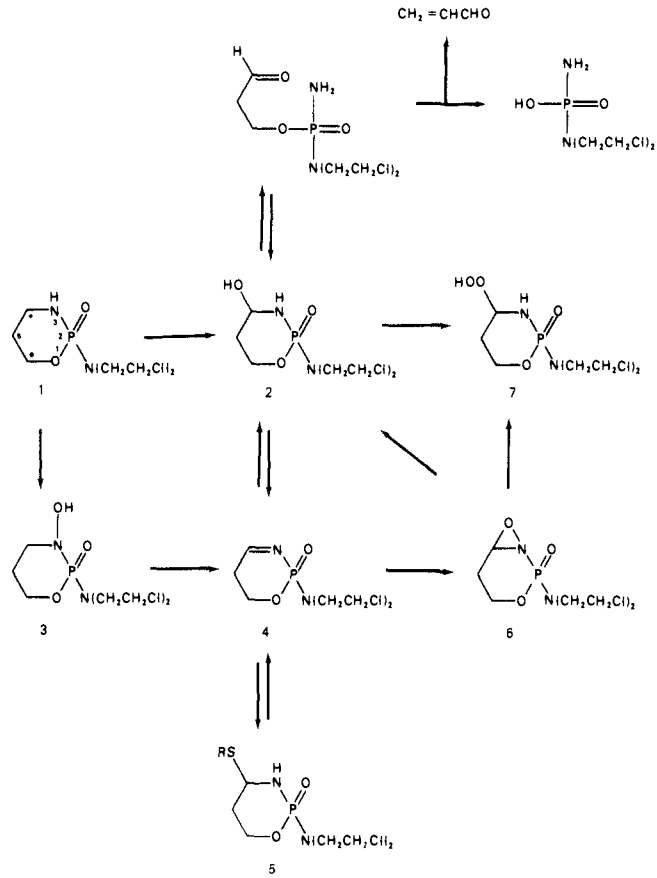
Hydrogenolysis of 3-(benzyloxy)cyclophosphamide (10) using Pd/C catalyst and ethyl acetate as solvent leads to the formation of 3-hydroxycyclophosphamide (3, ~20%) and cyclophosphamide (1, ~10%), accompanied by regioselective hydrogen-exchange reactions at the C-4 and C-5 positions in 3 and 1. A variety of oxidizing reagents and liver microsomal incubation failed to provide evidence (^{31}P NMR) for conversion of 1 into 3, whereas identical incubation of 3 led to its reduction to 1. Compound 3 is stable at pH 6.5–8.2, 37 °C, and exhibits anticancer activity comparable to 1 when tested against L1210 leukemia in mice. Data are discussed with regard to a previously reported suggestion that metabolism of 1 may involve oxidation to give 3 followed by rearrangement of 3 to 2.

Current theories regarding the oncostatic selectivity of cyclophosphamide (1) implicate a pivotal role for 4-hydroxycyclophosphamide (2), which is produced by liver microsomal oxidation of the parent prodrug.[2] The details of this initial enzymatic "activation" process are thus of considerable importance in the continuing search for cyclophosphamide analogues having improved anticancer properties.[3] Early mechanistic studies by Connors and co-workers[4] utilized cyclophosphamide-*4,4-d*$_2$ and mass spectroscopic analysis of the deuterated acrolein fragment (Scheme I) to determine indirectly that incubation of 1 with rat liver microsomes can lead to an ca. 90:10 ratio of C-4/C-6 hydroxylation, although additional evidence for the minor 6-hydroxy metabolite of 1 is still unavailable. Connors et al.,[4] also found that there is no significant aggregate isotope effect for the enzymatic processing of 1, relative to the *4,4-d*$_2$ substrate, which was in accord with the observation[4] of essentially equivalent activities for these two compounds against ADJ/PC6 tumor in mice. The apparent absence of a measurable isotope effect for liver microsomal hydroxylation of the C-4 position in 1 prompted the suggestion[4] that cleavage of the isotopically substituted bond is not rate limiting, as might be expected for an oxene insertion reaction.[5] An alternative mechanistic rationale was proposed,[4] involving initial hydroxylation of the N-3 position to give 3-hydroxycyclophosphamide (3) followed by an unspecified rearrangement of 3 to the 4-hydroxy isomer (2). It was noted[4] that similar hydroxylation of isophosphamide is precluded by the presence of a 2-chloroethyl substituent at the endocyclic nitrogen position, which suggests that in this molecule

Scheme I



direct C-4 hydroxylation is more likely. On the other hand, it is clear that such an argument by itself does not disprove the N-3 hydroxylation–rearrangement sequence for cyclophosphamide and analogous structures having an available endocyclic N–H bond.

With regard to possible reaction modes leading from 3 to 2, direct transposition of oxygen is highly unlikely based on the chemistry of hydroxylamines,[6] whereas the pre-

(1) This paper is part 4 of a series on "Synthesis and Antitumor Activity of Cyclophosphamide Analogues". For part 3, see V. L. Boyd, G. Zon, V. L. Himes, J. K. Stalick, A. D. Mighell, and H. V. Secor, *J. Med. Chem.*, **23**, 372 (1980).

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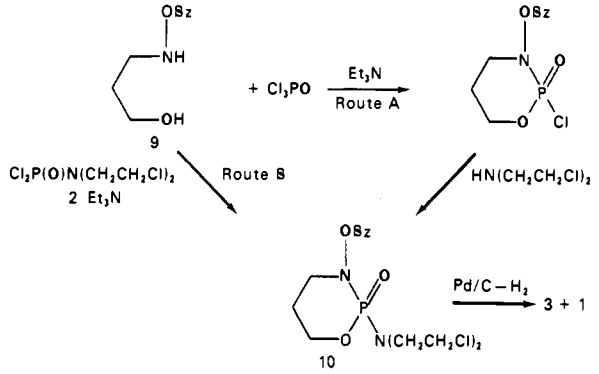
Scheme II



cedented[7] elimination of water from **3**, followed by the expected[8] hydration to give isomer **2**, implicates the intermediacy of iminophosphamide **4**, which is a structure that has also been proposed[2] in connection with the interconversion of **2** and 4-thio conjugates of cyclophosphamide (**5**, Scheme I). Another possibility for transformation of **3** into **2** involves oxidation[8] of the C=N moiety in **4** to give 3,4-epoxycyclophosphamide (**6**), followed by either enzymatic N–O bond reduction[9] to provide **2** or hydrolytic ring opening to afford 4-hydroperoxycyclophosphamide (**7**), a well-known[10] precursor of **2**, in vivo. Interestingly, it has been very recently reported[11] that synthetically derived **6** does produce **7** under mild hydrolytic conditions.

Any attempt to resolve these mechanistic questions which surround the intervention of 3-hydroxycyclophosphamide requires the synthesis and characterization of this material. Chemical methods for mimicking the enzymatic oxidation of cyclophosphamide have been widely investigated,[10-18] and in one of these studies it was suggested[13] that treatment of **1** with $FeSO_4$–H_2O_2 (Fenton's reagent) provides, inter alia, a substance which was identified as **3**. This structural assignment was later revised[14] to **2**; however, Struck et al.,[15] subsequently established that this product was 4-peroxycyclophosphamide (**8**), the anhydro dimer of **2**. We now report the first definitive synthesis of **3**, together with various results bearing upon the previously hypothesized[4] role of this compound in the metabolism of cyclophosphamide.

Results and Discussion

Regioselective oxidation of the N–H bond in cyclophosphamide is, in principle, the most direct synthetic route to **3**, although past studies with oxidizing agents such as O_3–H_2O_2,[16] $FeSO_4$–H_2O_2,[10c,15,17] and $KMnO_4$[18] have found, primarily, C-4 oxidation products. We obtained similar results with **1** and a variety of newer oxidizing reagents, viz., $NaWO_4$–H_2O_2, $KMnO_4$–crown ether, $K_2S_2O_8$, and *m*-chloroperbenzoic acid, and therefore focused upon 3-(benzyloxy)cyclophosphamide (**10**) as a precursor of **3** via selective reduction of the O–Bz bond. Montgomery and Struck[19] previously used route A in Scheme II for the preparation of **10**; however, Struck's efforts to generate **3** from **10** were unsuccessful.[20] A slightly different strategy (route B, Scheme II) for synthesizing **10** afforded analytically pure product in 30% yield, based on the amount of *O*-benzylhydroxylamine hydrochloride employed for the preparation of amino alcohol **9**. Initial attempts to debenzylate **10** with Pd/C, H_2 in methanol solvent were patterned after the hydrogenolysis conditions previously used for the synthesis of cyclophosphamide[21] and isophosphamide[22] enantiomers; however, after repeated failure to isolate a characterizable product, ethyl acetate was examined as an alternative reaction medium. When ethyl acetate solutions of **10** were shaken under medium pressures of H_2 (40–45 psi) for ca. 3–4 days at room temperature, using a relatively large (2-fold weight) excess of 10% Pd/C catalyst, a crystalline product (mp 146–148 °C dec) having an elemental composition consistent with **3** was obtained in 16–22% yield. The yield of this product could not be improved by variation of either the reaction time, the proportion of catalyst, or the source of the catalyst. Field-desorption mass spectroscopic measurements[20] confirmed that the molecular weight was equal to that of **3**, while the presence of a hydroxyl group was demonstrated by treatment of the hydrogenolysis product with Ac_2O–pyridine, which gave a single substance that was readily identified by GC–MS as an *O*-acetyl derivative of 3-hydroxycyclophosphamide. That this hydroxyl group is located at the 3 position of cyclophosphamide unambiguously follows from the observation of two-proton NMR multiplets for each of the ring carbons and, moreover, the observation of a ^{13}C NMR signal at 52.68[23] ppm for the C-4 carbon. As expected from the consideration of oxygen's inductive effect, this signal is moderately deshielded relative to the C-4 signal for **1** (41.44[24] ppm), whereas the oxygen-bearing C-4 nucleus in **6–8** (97.50,[11] 86.60,[15] and 86.85[15] ppm, respectively) is strongly deshielded. The remaining carbon signals for **3** and **1** have very similar chemical shifts. ^{31}P NMR data are also consistent with the presence of an electronegative hydroxyl group adjacent to phosphorus, since the ^{31}P signal for **3** (16.26[25] ppm) is

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(23) All ^{13}C NMR chemical shifts refer to $CDCl_3$ with internal Me_4Si.
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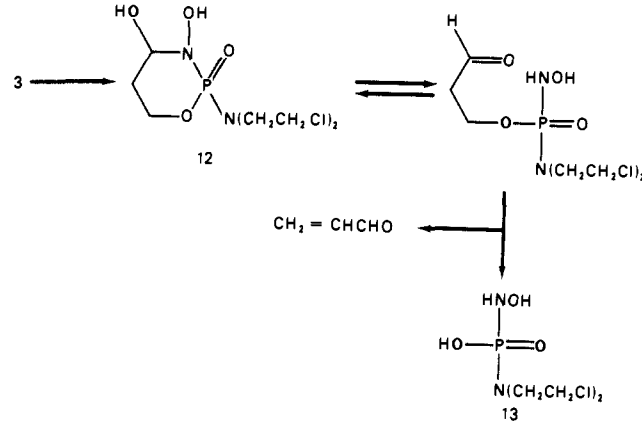
deshielded relative to the phosphorus in 1 (11.51 ppm) by an amount which is somewhat greater than that due to the less electronegative chlorine substituent in 3-chlorocyclophosphamide[26] (15.35 ppm).

Hydrogenolysis of compound 10 according to the above procedure also leads to the formation of cyclophosphamide, which was isolated in ca. 15% yield. Control experiments demonstrated that 3 undergoes extensive decomposition (ca. 80%) to unknown materials when subjected to reaction conditions equivalent to those used for its production from 10 and, likewise, affords ca. 5–15% yields of 1. These observations account for the low yield (ca. 20%) of 3 from 10 and suggest that the accompanying cyclophosphamide byproduct is formed, at least in part, through the intermediacy of 3; however, the proportion of direct N–OBz bond reduction leading from 10 to 1 is not known.[27]

Various approaches were used to evaluate the possible formation and significance of 3 in the metabolism of cyclophosphamide. Oxidizing agents such as $FeSO_4$–H_2O_2, $KMnO_4$, and O_3–H_2O_2, which have been employed to simulate enzymatic "activation" of 1, are known to lead to the formation of 4-hydroperoxycyclophosphamide (7), its anhydro dimer (8), and 4-ketocyclophosphamide (11). In view of the substantial ^{31}P chemical shift differences between 1 (11.51 ppm) and 3 (16.26 ppm) and these other products (7, 11.05 ppm; 8, 12.40 ppm; 11, 4.66 ppm), phosphorus NMR was utilized for analysis of crude product mixtures afforded by these and other (vide supra) oxidizing procedures, and in no instance was there evidence for a detectable amount (<1%) of the 3-hydroxy substance among the oxidized compounds.

A more realistic and frequently used method for studying cyclophosphamide "activation" involves the incubation of 1 with liver microsomes. High-field (121.5 MHz) ^{31}P NMR analysis of concentrated chloroform extracts of incubation mixtures containing 1, phenobarbital-induced rat liver microsomes, and cofactors failed to show the presence of even trace quantities (<0.01%) of 3. By way of contrast, identical incubation of synthetic 3 using the same liver microsome preparation led to ca. 10–15% conversion into 1 after 15 min at 37 °C, which is attributed to a reductase of the type known[9] to reduce hydroxylamino groups. Since this reductase could "recycle" 3 (vide infra) that is derived from liver microsomal N-hydroxylation of 1, it is not possible to argue against the latter process based on the absence of 3 in the cyclophosphamide incubation product mixture. Moreover, the existence of an efficient reductase for 3 suggests that the proposed[4] N-hydroxylation route to 2 may be a moot point if rearrangement of 3 to 2 is relatively slow. The chemical feasibility of etiher acid- or base-catalyzed rearrangement of 3 to 2 was thus examined by ^{31}P NMR monitoring of 3 in Tris buffer at 37 °C, and no reaction (<1%) was detected after 30 min at pH values of 6.5, 7.4, and 8.2.

Scheme III



Compound 3 was evaluated against L1210 leukemia in mice given an inoculum of 10^6 cells, and it was found that single ip injections in the 250–30 mg/kg dose range gave a maximum increased life span (ILS) of 128% at 150 mg/kg. By comparison, cyclophosphamide routinely gives ILS values of 75–100% and 150–200% at 100 and 200 mg/kg doses, respectively. The interpolated 125–150% ILS value for 1 at 150 mg/kg is essentially equal to that found for 3 at this dosage, which can be rationalized as follows: (1) compound 3 undergoes in vivo rearrangement to 2, (2) it is enzymatically reduced in the liver to give 1, which in turn is oxidized to 2,[28] or (3) it is oxidized at the C-4 position and the resultant metabolite, 3,4-dihydroxycyclophosphamide (12, Scheme III), leads to release of *N*-hydroxyphosphoramide mustard (13), a potential DNA cross-linking agent. While there is no presently available data which disfavors proposal 1, the second proposal is supported by the liver microsomal incubation experiments. Proposal 3, however, appears to be unlikely, since 13 has been found[29] to be ca. 100-times less reactive than phosphoramide mustard as an alkylating agent.

Conclusions

Reaction of cyclophosphamide (1) with a wide variety of chemical oxidizing agents as well as its incubation with rat liver microsomes demonstrate that 3-hydroxycyclophosphamide (3), if formed, does not accumulate in amounts which are detectable by ^{31}P NMR (<1% total intensity). The stability of 3 at pH 6.5–8.2, 37 °C, suggests that its formation in vivo would require the intervention of an enzyme for isomerization to 4-hydroxycyclophosphamide (2), while the anticancer activity exhibited by 3 against L1210 leukemia in mice, which is comparable to the activity of 1, can be accounted for by the proven liver

(25) All ^{31}P NMR chemical shifts refer to $CDCl_3$ with external 25% H_3PO_4 in D_2O.

(26) G. Özkan and G. Zon, unpublished results.

(27) The mechanism for conversion of 3 to 1 was briefly investigated to assess the possible intermediacy of 4 vs. direct N–O bond reduction. Exposure of 3 to the previously mentioned debenzylation conditions, using D_2 in place of H_2, afforded 1 having ca. 50 atom % D at the C-4 position, which is equal to the value expected for a reaction pathway wherein 3 is sequentially converted into 4 and then 1. Unfortunately, this interpretation is complicated first by the observation that recovered 3 showed ca. 60 atom % D incorporation at its C-4 position and second by the results of a control study with unlabeled 1, which revealed that ca. 20 atom % D was incorporated at C-4 in recovered 1. These competing modes of isotope incorporation before and after conversion of 3 into 1, together with the additional observation of ca. 20 atom % D at C-5 in all three of these samples, compel us to reserve final judgement on the intermediacy of dehydro compound 4. It is also worthwhile to note that ^{31}P NMR analysis of mixtures of 1, Pd/C catalyst, and a H_2 acceptor (excess cyclohexene) failed to show a new ^{31}P signal, indicating dehydrogenation of 1 to form either 4 or its 4,5-didehydro isomer. Mixtures of 3 and Pd/C catalyst in ethyl acetate under a N_2 atmosphere led to similar conclusions.

(28) If this reduction–oxidation sequence is operative, then the absence of L1210 anticancer activity found for 3-(benzyloxy)-cyclophosphamide (10, 250–50 mg/kg, single injections) implicates specific structural requirements for reductive conversion of 3-(alkoxy)- and 3-(aryloxy)cyclophosphamides into 1. Comparative studies of such compounds will be reported elsewhere.

(29) T. W. Engle and G. Zon, unpublished results. The investigation of 13 and other N-substituted derivatives of phosphoramide mustard will be reported elsewhere.

microsomal reduction of 3 to 1. In summary, it appears that the fate of the hypothetical 3-hydroxycyclophosphamide metabolite may be restricted to its return to cyclophosphamide.

Experimental Section

Ethyl acetate was purified by fractional distillation and Et_2O refers to freshly opened cans of anhydrous solvent. Palladium (10%) on charcoal powder (Pd/C) was obtained from Pfaltz and Bauer, Inc., and was used in conjunction with a Parr shaker–hydrogenator (500- and 50-mL vessels). D_2 (98 atom % D) was purchased from Roberts Oxygen Co. Melting points were obtained with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Inc. ^1H NMR spectra at 60 MHz were recorded in the continuous-wave mode on a Varian EM360-A spectrometer. ^1H NMR spectra at 100 MHz were obtained with a JEOL FX-100 spectrometer (quadrature phase detection) using 5-mm sample tubes. Conditions for the pulse Fourier transform spectra include 1-kHz spectral window, 8192 data points zero filled to 16 384, and a $\pi/2$ pulse of 18 μs. The delay time between pulse sequences was 10 s. Prior to Fourier transform, spectra were exponentially multiplied so as to result in an additional 0.25-Hz broadening in the frequency domain spectrum. ^{13}C NMR spectra at 25 MHz were recorded on a JEOL FX-100 spectrometer using 5-mm sample tubes. Pulse Fourier transform conditions include a 5-kHz spectral window, 8192 data points zero filled to 16 384, $\pi/2$ pulse of 12 μs, and high-power ^1H decoupling. The pulse repetition time was 10 s; spectra were broadened by 1 Hz due to exponential multiplication prior to Fourier transform. ^{31}P NMR spectra at 40.25 MHz were also obtained with the JEOL FX-100 instrument, using 10-mm sample tubes. Conditions for the pulse Fourier transform spectra include a 5-kHz spectral window, 8192 data points zero filled to 16 384, a $\pi/2$ pulse of 18 μs, and low-power ^1H decoupling. The pulse repetition time was 2 s; spectra were broadened by 1 Hz due to exponential multiplication prior to Fourier transform. ^{31}P NMR spectra at 121.5 MHz were recorded on a Bruker WM-300 spectrometer using 10-mm sample tubes. Sample conditions were a 5-kHz spectral window, 8192 data points zero filled to 16 384, a $\pi/2$ pulse of 20 μs, low-power ^1H decoupling, and a 2.5-s pulse repetition time; spectra were broadened by 1 Hz due to exponential multiplication prior to Fourier transform. ^2H NMR spectra at 41.45 MHz were obtained in the pulse Fourier transform mode utilizing a Bruker superconducting magnet with a "homebuilt" spectrometer and probe. A Nicolet 1180 computer system, including quadrature phase detection, was used for data collection and transformation. Sampling conditions included a 40-μs $\pi/2$ pulse, 8192 data points, a 4-kHz spectral window, and a 250-ms pulse repetition time. All ^1H, ^2H, and ^{13}C chemical shifts (δ, ppm) refer to $CDCl_3$ with internal Me_4Si as reference; ^{31}P chemical shifts in both $CDCl_3$ and D_2O refer to external 25% H_3PO_4 in D_2O, which was sealed in a capillary tube and positioned coaxially in the NMR sample using a vortex plug. NMR sample temperatures were measured by direct insertion of a small precalibrated thermometer before and after data accumulation. Analytical and preparative TLC employed 2.5 × 10 cm and 20 × 20 cm plates coated with 250-μm and 1-mm layers of silica gel GF, respectively; I_2 vapor was used for component visualization. The reported R_f values are approximate. Column chromatography utilized Baker 60–200 mesh silica gel. Mass spectra were obtained with an LKB Model 2091 GC–MS system.

***N*-(Benzyloxy)-*N*-(3-hydroxypropyl)amine (9).** NaOH (1 M) was added to a magnetically stirred and chilled mixture of *O*-benzylhydroxylamine hydrochloride (10.32 g, 65 mmol), water (40 mL), and Et_2O (40 mL) until a persistent pink color developed in the aqueous layer due to the presence of phenolphthalein. The Et_2O layer was removed and the aqueous layer was washed twice with Et_2O. The combined Et_2O solutions were dried with $MgSO_4$ and then concentrated on a rotary evaporator to give *O*-benzylhydroxylamine (7.38 g, 60 mmol, 93%) as a pure colorless oil: R_f 0.62 (Et_2O); ^1H NMR (60 MHz) δ 7.30 (s, 5, C_6H_5), 5.23 (s, 2, NH_2), 4.63 (s, 2, CH_2). A solution of this product (7.38 g, 60 mmol) and 3-bromopropanol (3.70 g, 27 mmol) in absolute EtOH (30 mL) was refluxed for 20 h and then concentrated on a rotary evaporator. EtOAc (200 mL) was added and the crystalline *O*-benzylhydroxylamine hydrobromide byproduct (26 mmol, 98%, pure by NMR) was collected for subsequent use as a source of the free amine. The filtrate was concentrated on a rotary evaporator and chromatography of the residue using Et_2O eluent gave 9 (3.72 g, 11.6 mmol, 44%) as a colorless oil, which was pure by TLC and NMR, and was used without further processing: R_f 0.30 (Et_2O); ^1H NMR (60 MHz) δ 7.33 (s, 5, C_6H_5), 4.70 (s, 2, CH_2), 4.00 (br s, 2, NH and OH), 3.68 (t, 2, CH_2OH), 3.07 (t, 2, CH_2N), 1.72 (m, 2, $CH_2CH_2CH_2$).

3-(Benzyloxy)-2-[bis(2-chloroethyl)amino]-2*H*-1,3,2-oxazaphosphorinane 2-Oxide [3-(Benzyloxy)cyclophosphamide, 10]. A solution of freshly recrystallized bis(2-chloroethyl)-phosphoramidic dichloride[30] (1.71 g, 6.6 mmol) in EtOAc (10 mL) was added to a solution of 9 (1.19 g, 6.6 mmol) and Et_3N (1.84 mL, 13.2 mmol) in EtOAc (10 mL), and the mixture was stirred for 7 days at room temperature before removal of Et_3N·HCl by suction filtration and then concentration of the filtrate on a rotary evaporator. The residual oil was purified by column chromatography using 1:1 $CHCl_3$–EtOAc eluent, which afforded 10 (1.76 g, 4.8 mmol, 73%) as a pale yellow oil: R_f 0.45 (1:1 $CHCl_3$–EtOAc); ^1H NMR (60 MHz) δ 7.33 (s, 5, C_6H_5), 4.73 (s, 2, $CH_2C_6H_5$), 4.52–3.88 (m, 2, CH_2CH_2O), 3.88–2.82 [m, 10, $N(CH_2CH_2Cl)_2$ and C-4 CH_2], 1.98–1.72 (m, 2, $CH_2CH_2CH_2$). Anal. ($C_{14}H_{21}N_2O_3PCl_2$) C, H, N.

2-[Bis(2-chloroethyl)amino]-3-hydroxy-2*H*-1,3,2-oxazaphosphorinane 2-Oxide (3-Hydroxycyclophosphamide, 3). A suspension of Pd/C (2.34 g) in EtOAc (35 mL) containing 10 (1.17 g, 32 mmol) was shaken in an atmosphere of H_2 at 40–45 psi for 3 days at room temperature. After solid material was removed by gravity filtration through a glass-fiber filter, the solution was concentrated on a rotary evaporator and the residual oil was crystallized from EtOAc by the addition of Et_2O. Product 3 (195 mg, 0.7 mmol, 22%) was thus obtained as a fine white powder: mp 146–148 °C dec; R_f 0.25 (EtOAc); ^1H NMR (100 MHz) 4.23 (m, 2, CH_2O), 3.55–3.22 [m, 10, $N(CH_2CH_2Cl)_2$ and C-4 CH_2], 2.86 (br s, 1, OH), 1.95–1.85 (2 m, 2, $CH_2CH_2CH_2$); ^{31}P NMR (40.25 MHz) δ 16.26 vs. δ 11.51 for 1. ^{13}C NMR (25 MHz, corresponding values for 1[24] are given in parentheses) δ 67.47 (67.76), C-6; 52.68 (41.44), C-4; 49.40 (48.92), NCH_2CH_2Cl; 42.23 (42.32), NCH_2CH_2Cl; 27.20 (25.82), C-5. Field-desorption MS (Varian 311A) (Me_2SO), *m/e* 276 (M^+) and 277 $(M+1)^+$ only.[20] Reaction of 3 (1–2 mg) in $CHCl_3$ (200 μL) with Ac_2O (100 μL) and pyridine (100 μL) at 50 °C for 1 h gave the *O*-Ac derivative: GC–MS *m/e* 319 [$(M + 1)^+$].

Reaction of 3 and 1 with Pd/C and D_2 and Related Studies. A suspension of Pd/C (60 mg) in EtOAc (5 mL) containing 3 (30 mg, 0.11 mmol) was shaken in an atmosphere of D_2 at 40–45 psi for 4 days at room temperature. After solid material was removed by gravity filtration through a glass-fiber filter, the solution was concentrated on a rotary evaporator and the residue was then subjected to preparative TLC using EtOAc eluent. Bands corresponding to 3 (R_f 0.25) and 1 (R_f 0.15) were collected and thoroughly washed with acetone to afford crystalline 3 (1.6 mg, 0.006 mmol, 6%) and oily 1 (2.5 mg, 0.01 mmol, 10%). Each sample was quantitatively transferred into a specific volume of $CDCl_3$ (1.00 mL) for high-field (41.45 MHz) ^2H NMR analysis. Signal intensities for the C-4 and C-5 deuterons in 3 and 1 were compared to that of $CDCl_3$ by using the cut and weigh method. Since the $CDCl_3$ signal intensity corresponds to 1.94×10^{-3} "mmol" of D, the intensity ratios for C-4 $^2H/CDCl_3$ and C-5 $^2H/CDCl_3$ provide a measure of mmol of D at these positions for comparison with the mmol amount of sample used in each case. Incorporation of ^2H found for 3: C-4, 64%; C-5, 17%. Incorporation of ^2H found for 1: C-4, 54%; C-5, 21%.

Repetition of the above experiment using 1 (360 mg, 1.4 mmol) and Pd/C (720 mg) in EtOAc (20 mL) led to preparative TLC (9:1 $CHCl_3$–MeOH) recovery of 1 (R_f 0.65) in 14% yield (0.195 mmol) for ^2H NMR analysis of this sample in $CDCl_3$ (1.00 mL) as described above. Incorporation of ^2H found for 1: C-4, 25%; C-5, 19%.

A solution of anhydrous 1 (250 mg, 0.96 mmol) in EtOAc (10 mL) and cyclohexene (9 mL), which had been fractionally distilled from P_2O_5 under N_2, was shaken with Pd/C (500 mg) in an atmosphere of N_2 at 40–45 psi for 3 days at room temperature.

(30) S. M. Ludeman and G. Zon, *J. Med. Chem.*, 18, 1251 (1975).

Solid material was removed by gravity filtration through a glass-fiber filter, using a blanket of N_2, and the filtrate was analyzed by ^{31}P NMR without further processing. The only phosphorus signal observed was that of 1.

The aforementioned experiment with 3 and Pd/C in EtOAc was repeated exactly as described, except for the substitution of 1 atm of N_2 for pressurized D_2. After solids were removed by filtration under a blanket of N_2, ^{31}P NMR analysis of the filtrate without further processing showed only the presence of 3.

Attempted Oxidations of 1 to Form 3. O_3–H_2O_2. A solution of 1·H_2O (2.14 g, 7.7 mmol) in water (22.5 mL) and acetone (11.5 mL) containing H_2O_2 (1.9 mL of 30% H_2O_2 in water) was cooled in an ice bath, and O_3 was then bubbled through the reaction mixture at a rate of 5 g/h (OREC 03V5-0 ozonator). After 1 h, more H_2O_2 (1.9 mL of 30% reagent) was added together with enough acetone (ca. 6 mL) to obtain the original total volume. This procedure was repeated after a second 1-h period, and after a total ozonation time of 3 h, the acetone was removed in vacuo at 5 °C. The aqueous solution was immediately analyzed by ^{31}P NMR (40.25 MHz) and signals were identified by comparisons with authentic materials: 1 (15.90 ppm, 53%), *cis*- and *trans*-7 (11.74 and 11.52 ppm, 15 and 7%, respectively), and 11 (8.24 ppm, 22%). An unidentified minor product (20.23 ppm, 3%) was also present; however, compound 3 (17–18 ppm) was not detectable (<0.5%).

$FeSO_4$–H_2O_2–EDTA. A mixture of 1·H_2O (1.39 g, 5 mmol), $FeSO_4$ (1.52 g, 10 mmol), EDTA·$2H_2O$ (5.36 g, 20 mmol), and H_2O_2 (7 mL of 30% reagent) in water (200 mL) was stirred for 5 days at room temperature. The residue obtained by rotary evaporation of solvent after $CHCl_3$ extraction (100 mL, twice) was analyzed by ^{31}P NMR (40.25 MHz), which showed the presence of 8 (12.40 ppm, 30%), 11 (4.66 ppm, 60%), and two unidentified components (18.07 and 11.70 ppm, ca. 10%); a signal for 3 (16.26 ppm) was not detected (<0.5%). A control experiment in which 3 was subjected to the above reaction conditions demonstrated (TLC) that this compound underwent ca. 50–75% decomposition. Repetition of the Fenton oxidation of 1·H_2O with extraction after only 3 h of reaction at room temperature gave a low-conversion sample which was void of detectable 3 (^{31}P NMR, <0.5%).

$KMnO_4$. Oxidation of 1·H_2O according to the procedure of Cox et al.,[18c] followed by ^{31}P NMR (40.25 MHz) and TLC analysis of the concentrated $CHCl_3$ extract, failed to provide evidence for 3. Repetition of this experiment using 1:1 $KMnO_4$–18-crown-6 gave the same result.

$NaWO_4$–H_2O_2. The following procedure is analogous to those used for the preparation of stable nitroxide spin-labels.[31] A solution of 1·H_2O (20 mg, 0.07 mmol) in D_2O (1 mL) was combined with a solution of EDTA (3.4 mg, 0.01 mmol) in D_2O, and H_2O_2 (70 μL of 30% reagent) and $NaWO_4$ (68 mg, 0.25 mmol) were then added. The reaction mixture was periodically examined by ^1H NMR (60 MHz), and after 5 days at room temperature, no change in the spectrum was evident.

$K_2S_2O_8$. An unbuffered solution of 1·H_2O (26 mg, 0.1 mmol) in D_2O containing 1 equiv of $K_2S_2O_8$ was periodically examined by ^1H NMR (220 MHz[32]), and after 3 days at room temperature, the spectrum was essentially identical with that of hydrolyzed starting material.[32]

***m*-Chloroperbenzoic Acid.** A solution of 1·H_2O (208 mg, 0.75 mmol) and technical-grade (85%) *m*-chloroperbenzoic acid (202 mg, 1 mmol) was refluxed for 3 h. Analytical TLC (1:1 acetone–EtOAc) of the reaction mixture showed mainly 1 and a relatively weak intensity spot with approximately the same R_f as 3; however, ^{31}P NMR (40.25 MHz) analysis of the concentrated crude reaction mixture demonstrated that this minor product was not due to 3, based on the absence of a detectable (<0.5%) signal at 16.26 ppm.

Incubations with Rat Liver Microsomes. The following procedure is essentially the same as that previously used in these laboratories for "activation" of the enantiomers of 1 with rat liver microsomes.[21b] Two male CD rats (ca. 150 g) were given ip injections of phenobarbital at doses of 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 16 h, beginning the evening of the 3rd day, and were then sacrificed. Liver tissue (22 g) was processed as detailed by Sladek,[33] and the resultant microsomal pellet was resuspended in cold 1.15% KCl so that 1 mL of the suspension contained material derived from 1 g of wet liver tissue. Cyclophosphamide monohydrate (11.2 mg, 0.040 mmol) and 3 (9.8 mg, 0.036 mmol) were separately dissolved in a solution of NADP sodium salt (0.20 mL of 0.02 M), glucose 6-phosphate disodium salt (0.20 mL of 0.20 M), phosphate buffer (2 mL of 0.20 M, pH 7.4), $MgCl_2$ (0.20 mL of 0.10 M), and 1.15% KCl (5.4 mL), which had been adjusted to give a final pH of 7.4. These solutions were equilibrated for 10 min at 37 °C in a shaker incubator and were then charged with glucose-6-phosphate dehydrogenase (0.11 mL of cold suspension equal to 4 enzyme units), followed by the cold liver microsomal suspension (2 mL). After 15 min, each incubation mixture was diluted with water (50 mL) and then vigorously extracted with $CHCl_3$ (25 mL, twice). The extracts were dried ($MgSO_4$) and concentrated in vacuo to afford 9.9 mg (89%) of residual material from 1 and 10 mg (102%) of residual material from 3. Each of these samples were dissolved in $CDCl_3$ (2 mL) and were kept refrigerated until analysis by ^{31}P NMR (121.5 MHz). No 3 (<0.01%) was detected in the sample derived from 1, whereas the sample derived from 3 showed the presence of 1 (11.51 ppm) and 3 (16.26 ppm) in a relative ratio of 12:88.

Anticancer Screening Tests. Female BD_2F_1 mice approximately 8–9 weeks old were given an inoculum of L1210 cells (10^6), and after 5 days, the test group received single ip injections of 3 in saline at doses of 150, 120, 90, 60, and 30 mg/kg. The first three dose levels resulted in increased life spans (ILS) of 128, 89, and 75%, respectively, compared to the controls. In this test system, routinely obtained data for 1 are as follows: 250 mg/kg, indefinite survival (no tumor over a 60-day period); 200 mg/kg, 150–200% ILS; 100 mg/kg, 75–100% ILS. Higher doses for 3 were precluded by its limited solubility in saline. Suspensions of 3 in Tween-80 diluted with saline gave the following results: 250 mg/kg, 50% ILS; 200 mg/kg, 30% ILS; 150 mg/kg, 55% ILS. Solutions of 10 in saline showed no effect when given at doses of 250, 200, 150, 100, and 50 mg/kg.

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