Synthesis and Evaluation of 3-Halocyclophosphamides and Analogous Compounds as Novel Anticancer "Pro-Prodrugs"

GERALD ZON *x, SUSAN MARIE LUDEMAN *, GÜNAY ÖZKAN *, SRINIVASAN CHANDRASEGARAN ‡, CHARLES F. HAMMER ‡, RUTH DICKERSON §, KAZUTAKA MIZUTA ¶, and WILLIAM EGAN **

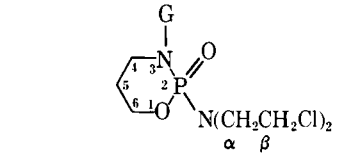
Received March 19, 1982, from the *Department of Chemistry, The Catholic University of America, Washington, DC 20064, ‡Department of Chemistry, Georgetown University, Washington, DC 20057, §The Johns Hopkins Oncology Center, Baltimore, Maryland 21205, ¶Otsuka Pharmaceutical Co., Ltd., Osaka, Japan, and **Division of Biochemistry and Biophysics, Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland 20205. Accepted for publication June 11, 1982.

Abstract □ 3-Fluoro-, 3-chloro-, and 3-bromocyclophosphamide were prepared from the reaction of trifluoromethylhypofluorite, sodium hypochlorite, and bromine with the anticancer drug cyclophosphamide. Treatment of *cis*- and *trans*-4-phenylcyclophosphamide and 5,6-benzocyclophosphamide with sodium hypochlorite afforded *cis*- and *trans*-3-chloro-4-phenylcyclophosphamide and 3-chloro-5,6-benzocyclophosphamide, respectively. ^{31}P-NMR spectroscopy was used to study the reactivity of these compounds: the fluoro derivative was reduced to cyclophosphamide on incubation with mouse liver slices, and the reactivity order for sulfhydryl-induced reduction of the 3-halocyclophosphamides was Br ≅ Cl ≫ F. Compared with the therapeutic efficacy of cyclophosphamide against L-1210 and P-388 cancers in mice, 3-fluoro- and 3-chlorocyclophosphamide were less active, although the fluoro derivative was more efficacious than the 3-chloro compound. The individual *R* and *S* enantiomers of 3-chlorocyclophosphamide, prepared from (*S*)- and (*R*)-cyclophosphamide, respectively, showed no significant difference in therapeutic activity in the P-388 test system.

Keyphrases □ Cyclophosphamide—synthesis of *N*-halogenated derivatives, NMR spectroscopic characterization, therapeutic efficacy in mouse cancers □ Synthesis—of *N*-halogenated derivatives of cyclophosphamide, NMR spectroscopic characterizations, therapeutic efficacy in mouse cancers □ Antineoplastic agents—cyclophosphamide and *N*-halogented derivatives, synthesis and NMR characterization, therapeutic efficacy in mouse cancers

The *N*,*N*-bis(2-chloroethyl)amino functionality has been used frequently in the design of antineoplastic agents (1) and prodrugs (1, 2). Studies of such prodrugs by Zon *et al.* (3–8) have included labile precursors of the well-known anticancer agent cyclophosphamide (I) (9). Since I functions as a prodrug (2, 9), these *in vivo* precursors of cyclophosphamide may be viewed as "pro-prodrugs." For example, 3-(*tert*-butyldimethylsilyl)cyclophosphamide (II) can release I by spontaneous hydrolysis (5), and 3-hydroxycyclophosphamide (III) can afford I by reductive cleavage of the N—O bond (6). While II proved to be inactive against L-1210 leukemia in mice (5), the *in vivo* conversion of III to I was evidenced by comparable antileukemic activities for III and I in the L-1210 system and by the rapid formation of I on incubation of III with liver microsomes (6).

A variety of other biologically reducible linkages (10–14) may be incorporated at the N-3 position of the cyclophosphamide ring system; however, the nitrogen–halogen bond is particularly interesting, since it apparently represents a new moiety for the reversible modification of N—H groups in drugs (11–15). In addition, the N—F modification, exemplified by 3-fluorocyclophosphamide (IV), could allow metabolic studies by ^{19}F-NMR spectroscopy, as with fluorine-labeled amino acids (16). Reports dealing with other types of halogenated cyclophosphamides [*e.g.*, *cis*- and *trans*-5-fluoro- (17, 18), 5-chloro- (17), and 5-bromocyclophosphamide (19)] have been published; however, unlike the *N*-halo series, the drug-design concepts were predicated on the *in vivo* stability of carbon–halogen bonds. 3-Fluoro- (IV) and 3-bromocyclophosphamide (VI) have not, to our knowledge, been previously synthesized; 3-chlorocyclophosphamide (V) has been mentioned only briefly in the literature (20). The present report describes the preparation of IV–VI and several 3-chloro analogues of I (X–XII). Data are also presented regarding the chemical charactristics and anticancer activity of these intended "pro-prodrugs."



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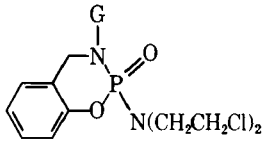
I: H
II: $Si(CH_3)_2C(CH_3)_3$
III: OH
IV: F
V: Cl
VI: Br

I → (CF_3OF, NaOCl, Br_2)



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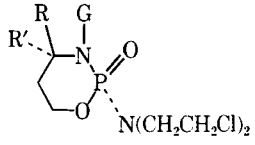
VII: H
X: Cl



	R	R′	G
VIII:	C_6H_5	H	H
XI:	C_6H_5	H	Cl
IX:	H	C_6H_5	H
XII:	H	C_6H_5	Cl

EXPERIMENTAL

Commercially available racemic[1] cyclophosphamide monohydrate (I·H_2O) was used as received; however, when necessary, I·H_2O was converted to anhydrous I as previously described (21). Enantiomerically pure

[1] Unless specified otherwise, all chiral compounds were used as their racemates.

0022-3549/83/0600-0687$01.00/0
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Table I—^{13}C-NMR Parameters for Cyclophosphamide (I) and Its Analogues (III–XII) [a]

Compound	C-4	C-5	C-6	C-α	C-β
I	41.44(3.7)	25.75(4.9)	67.66(6.1)	48.78(4.9)	42.28
III	52.68	27.20	67.47(6.1)	49.40(4.9)	42.23
IV	54.14(3.7)[b]	27.63[c]	68.73(6.1)	49.54(2.5)	42.59
V	56.90	28.61	67.54(6.1)	49.44(4.9)	41.79
VI	58.62(3.7)	29.53	67.61(6.1)	49.62(3.7)	41.93
VII[d]	43.25	118.8(7.3)	172.0(10)	49.10(3.7)	42.22
X[e]	49.32	119.0(5.5)	172.0(10)	49.06(3.0)	41.84
VIII[f]	56.93	33.61(7.9)	65.41(6.7)	48.78(4.2)	42.11
XI[g]	70.00	35.50(3.7)	62.91(6.1)	50.02(4.3)	41.95
IX[h]	57.15	34.78	66.36	48.87	42.36
XII[i]	69.7	37.9	65.8	49.8	41.9

[a] The tabulated chemical shifts (δ) refer to internal tetramethylsilane in deuterochloroform solvent, except for III and IV, which were dissolved in deuterium oxide and acetone-d_6, respectively. The values in parentheses are ^{13}C—^{31}P coupling constants in Hz-units. The carbon positions are as shown in the structure for I. [b] J_{CF} = 11.6 Hz. [c] J_{CF} = 9.8 Hz. [d] Other aromatic carbons at δ123.8, 126.5, and 128.8. [e] Other aromatic carbons at δ124.2, 130.6, and 135.9. [f] Other aromatic carbons at δ126.2, 127.8, and 128.7. [g] Other aromatic carbons at δ127.0, 128.0, and 128.8. [h] Other aromatic carbons at δ126.1, 128.3, 129.0, and 146.9. [i] Dilute sample; δ values are approximate and coupling constants were not measured, due to added line broadening.

samples of (+)-(*R*)- and (−)-(*S*)-I·H_2O were used as received[2]. The syntheses of 5,6-benzocyclophosphamide (VII), *cis*-4-phenylcyclophosphamide (VIII), and *trans*-4-phenylcyclophosphamide (IX) were reported elsewhere (22, 23), as were the details of the 40.25-MHz ^{31}P-NMR[3] and 25-MHz ^{13}C-NMR[3] measurements (6). Analogous procedures for data acquisition and processing were employed for obtaining 36.23-MHz[4] and 121.5-MHz[5] ^{31}P-NMR, 22.49-MHz[4] and 75.47-MHz[5] ^{13}C-NMR, 89.55-MHz ^{1}H-NMR[4] and 93.65-MHz ^{19}F-NMR[3] spectra. Chemical shift (δ, ppm) references were as follows: phosphorus-31, 25% (v/v) H_3PO_4 in deuterium oxide (external); carbon-13, tetramethylsilane (internal); fluorine-19, hexafluorobenzene (internal). Electron-impact (70 eV) mass spectra[6] (MS) were recorded with samples introduced *via* a solids' inlet probe, which was heated from ambient temperature to 100°. TLC plates had 0.25-mm coatings of silica gel and were developed by exposure to iodine vapor, which led to either absorptive (brown) or repellent (white) spots.

3-Fluorocyclophosphamide (IV)—A magnetically stirred and dry nitrogen-purged solution of anhydrous I (3.64 g, 13.9 mmoles) in dry methylene chloride (50 ml) was cooled to −78°. Trifluoromethylhypofluorite gas[7] (4.65 mmoles) was slowly introduced over a period of 3 hr into the nitrogen flush line *via* a Y-tube connected with tubing[8] to a cylinder, using a series of valves and a metering gauge that are described elsewhere (24). The nitrogen from the reaction flask was passed into a bubble cylinder containing an alkaline solution of potassium iodide to remove all by-products[9] and unreacted trifluoromethylhypofluorite. After the addition of the hypofluorite, the solution was allowed to gradually warm to room temperature (overnight) using a very slow nitrogen flush. The reaction mixture was filtered, and an aliquot (0.2 ml) of the filtrate was diluted with deuterochloroform (1.8 ml) prior to ^{31}P-NMR analysis. The remaining solution was concentrated on a rotary evaporator, without heating[10], and the resultant oil was chromatographed on a column (2.2 cm × 25 cm) of silica gel using ethyl acetate as the eluent. Compound IV (R_f 0.5, ethyl acetate) was eluted with 85–135 ml of solvent and was obtained as a pale-yellow oil (252 mg, 20% yield); ^{31}P-NMR (deuterium oxide): δ 16.17, J_{PF} = 22 Hz; ^{19}F-NMR (deuterochloroform): δ 83.27, J_{FP} = 22 Hz; ^{13}C-NMR, *cf.* Table I; MS, *m/z* 278, 280, and 282 (M^+, 2 Cl), 229 and 231 (base-peak cluster, $M-CH_2Cl$).

3-Chlorocyclophosphamide (V)—A magnetically stirred solution of I·H_2O (270 mg, 0.97 mmole) in chloroform (15 ml) was cooled with an ice-water bath, and an aqueous solution of sodium hypochlorite[11] [15 ml, 5.25% (w/w) NaOCl, 10.6 mmoles] was added; after 4 hr, the water bath was removed. Aliquots (1 ml) of the chloroform layer were periodically removed, diluted with deuterochloroform (1 ml), and the relative signal intensities for I (δ12.10) and the only detectable product (δ15.95) were measured by ^{31}P-NMR: 50:50, 2 hr; 25:75, 4 hr; 0:100, 18 hr. After 18 hr of stirring, TLC (ethyl acetate) of the chloroform layer confirmed the absence of I (R_f 0.1) and a single spot was evident (R_f 0.5). The 18-hr NMR sample and the remainder of the chloroform layer (13 ml) were combined, washed with water (15 ml) twice, dried with sodium sulfate, and then concentrated *in vacuo* without heating. Based on elemental composition, the resultant oil (280 mg, 87% yield) was identified as V·HCl, which was converted to V (^{13}C-NMR, Table I) by dissolving in chloroform and stirring with solid potassium carbonate.

Anal.—Calc. for $C_7H_{15}Cl_4N_2O_2P$: C, 25.32; H, 4.55; N, 8.44; Cl, 42.71. Found: C, 26.74; H, 4.71; N, 8.55; Cl, 43.12.

Application of the above procedure to (*R*)- and (*S*)-I·H_2O (2-g scale) gave (*S*)- and (*R*)-V, respectively[12], which were identified and checked for purity by TLC.

3-Bromocyclophosphamide (VI)—Bromine (0.135 ml, 2.6 mmoles) was added in one portion to a magnetically stirred, cold (5°) mixture of I·H_2O (0.70 g, 2.5 mmoles) and potassium carbonate (0.34 g, 2.5 mmoles) in methylene chloride (10 ml). After being stirred for 2 hr, the mixture was filtered and the solvent was removed on a rotary evaporator, without heating, and the residue was then placed under a high vacuum. The resultant orange-colored oil was free of TLC-detectable I (R_f 0.1, ethyl acetate), and gave rise to a single spot (R_f 0.4, ethyl acetate) which was identified by ^{13}C-NMR (Table I) as VI (0.88 g, 100% yield).

3-Chloro-5,6-benzocyclophosphamide (X)—Compound VII (31 mg, 0.1 mmole) in chloroform (3 ml) was treated with aqueous sodium hypochlorite (3 ml) as described above for the preparation of V. After stirring at room temperature overnight, the separated chloroform layer was washed with water (6 ml) twice, dried with magnesium sulfate, and then concentrated *in vacuo* to give X as an unstable oil (26 mg, 80% yield): TLC (ethyl acetate): R_f 0.8 for X *versus* R_f 0.4 for VII; see Table I for ^{13}C-NMR data for X *versus* VII. After 24 hr at room temperature, the two sets of protonated aromatic carbon signals for X and VII had relative intensities of ~67% and ~33%, respectively, indicating spontaneous decomposition.

***cis*- and *trans*-3-Chloro-4-phenylcyclophosphamide (XI) and (XII)**—A solution of VIII (28 mg, 0.08 mmole) in chloroform (3 ml) was stirred with aqueous sodium hypochlorite (6 ml) at room temperature for 7 days. Workup, as described above for X, afforded XI as an oil (17.4 mg, 59% yield) that was free of TLC-detectable VIII (R_f 0.5, ethyl acetate) and was seen as a single, faster eluting spot (R_f 0.9, ethyl acetate). A scaled-down version of this reaction using IX (3.5 mg, 0.01 mmole) gave XII (3.5 mg, 94% yield), which showed one spot on TLC (ethyl acetate), R_f 0.8 *versus* R_f 0.3 for IX. Compounds XI and XII were identified by ^{13}C-NMR comparisons with VIII and IX, respectively (Table I).

Incubation Studies—Female Balb/C mice (~25 g) were sacrificed by cervical dislocation. The livers were immediately removed for manual slicing, and the slices were stored temporarily in saline at 5°. Separate saline (10 ml) solutions of III (10 mg, 0.036 mmole) and IV (10 mg, 0.036 mmole), obtained by sonication, were equilibrated at 37° using open 50-ml Erlenmeyer flasks and a shaker incubator, and ~1- and 3-g portions of the liver slices were then added. After 10 min, each of the incubation mixtures was vigorously stirred with chloroform (25 ml) for 20 min; the separated organic layers were then dried with magnesium sulfate, filtered, and concentrated on a rotary evaporator. Both samples derived from III weighed ~10 mg, whereas the 1-g and 3-g incubations with IV afforded ~4 and 2 mg of recovered material, respectively. Each of the extracts was

[2] Otsuka Pharmaceutical Co., Ltd. For (*R*)-I·H_2O (mp 68°), $[\alpha]_D^{25}$ = 2.40 (c = 10, methanol); for (*S*)-I·H_2O (mp 68°), $[\alpha]_D^{25}$ = −2.47 (c = 10, methanol).

[3] FX-100 spectrometer, *JEOL U.S.A.*, Inc.

[4] FX-90Q spectrometer, JEOL U.S.A., Inc.

[5] WM-300 spectrometer, Bruker Instruments, Inc.

[6] Model JMS-01SG-2 spectrometer, JEOL U.S.A., Inc.

[7] PCR Research Chemicals, Inc.

[8] Teflon.

[9] Carbonyl difluoride and hydrofluoric acid.

[10] Above ~50°, product IV underwent relatively rapid decomposition. Pure IV was obtained from partially decomposed samples by extraction into methylene chloride.

[11] *Commercially available bleach.*

[12] On conversion of I to V, the *R* and *S* descriptors for the absolute configuration of I respectively change to *S* and *R* for V, although the stereochemistry about phosphorus is preserved.

Table II—Anticancer Screening Data for Analogues of Cyclophosphamide Against Mouse L-1210 Lymphoid Leukemia

Compound[a]	Mouse Type	Injection Data	Dose, mg/kg	ILS[b], %	T/C[b], %
(*R*,*S*)-IV[c] (in Vehicle 1)	BDF_1 (*n* = 7)	10^5 cells/mouse on day 2	200	30.6	
			100	8.0	
			50	9.6	
(*R*,*S*)-I·H_2O[c] (concurrent positive control)			100	62.5	
(*R*,*S*)-IV[d] (in vehicle 2)	BD_2F_1 (*n* = 4)	10^6 cells/mouse on day 5	250	—[g]	
			200	—	
			150	86	
			100	35	
			50	14	
(*R*,*S*)-I·H_2O[d] (concurrent positive control)			250	All cured	
			200	175 ± 25	
			100	87 ± 12	
(*R*,*S*)-V[e] (in vehicle 3)	C57BL/6 (*n* = 6)	10^6 cells/mouse on day 1	500		129
			250		109
			125		96
			63		96
			31		93
(R,S)-I·H_2O[e] (concurrent positive control)			500		112[h]
			250		282
			125		173
			63		124
(*R*)-V[c] (in vehicle 4)	BDF_1 (*n* = 7–8)	10^5 cells/mouse on day 2	339	15.9	
			226	9.5	
			113	8.1	
(*S*)-V[c] (in vehicle 4)			339	25.0	
			226	17.7	
			113	5.6	
(*R*,*S*)-I·H_2O[c] (concurrent positive control)			300	81.6	
			200	76.5	
			100	49.3	
XI–XII (~1:1 in vehicle, 3)[e]	CD_2F_1 (*n* = 6)	10^6 cells/mouse on day 1	350		110
			175		106
			88		103
			44		106
VIII–IX (~1:1 in vehicle 3)[e,f]	CD_2F_1 (*n* = 6)	10^6 cells/mouse on day 1	500		204
			250		122
			125		120
			63		105

[a] Vehicle key: (1) corn oil; (2) ethanol–propylene glycol–water (30:30:40 v/v/v); (3) aqueous ethanol with polyoxyethylene sorbitan *monooleate*; (4) 1% (v/v) carboxymethylcellulose in water. [b] (ILS) increased life span; (T/C) test/control. [c] Conducted at Otsuka Pharmaceutical Co. Ltd., Osaka, Japan. [d] Conducted at Johns Hopkins Oncology Center, Baltimore, Md. [e] Conducted at the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. [f] Taken from Ref. 23. [g] — Unacceptable level of toxicity. [h] Four of six toxicity-day survivors.

analyzed by 40.25-MHz ^{31}P-NMR using deuterochloroform solvent (1.8 ml), a $\pi/2$ pulse, 2-sec pulse-repetition time, and nuclear Overhauser enhancement. The presence of I was established by TLC (ethyl acetate) and addition of authentic I·H_2O (^{31}P-NMR). Integrated signal intensities were measured by the "cut-and-weigh" method.

Partition Coefficients—The ^{31}P-NMR spectrum of 27.9 m*M* I in water [5% (v/v) ^2H_2O] was obtained using a standard set of acquisition parameters [80 pulses ($\pi/2$), no nuclear Overhauser enhancement, and a 10-sec pulse-repetition time] and a standard set of display parameters. Comparison of the resultant signal intensity [(peak height) × (width at half-height) = 283 units] with the signal intensity (153 units) similarly obtained with a saturated solution of V in water gave an initial value of 15.1 m*M* V. A 2-ml aliquot of 27.9 m*M* I was vigorously stirred (25°, 5 min) with 1 ml of 1-octanol, and a 2-ml aliquot of 15.1 m*M* V was extracted with 0.1 ml of 1-octanol in exactly the same manner. The ^{31}P-NMR spectrum for each of the separated aqueous layers was recorded as described above, and the signal intensities (61.2 units for I, 28.5 units for V) were used to compute the concentration of I and V in the water and octanol layers: $[I]_{water}$ = 6.02 m*M*, $[I]_{octanol}$ = 43.71 m*M*, $[V]_{water}$ = 2.81 m*M*, and $[V]_{octanol}$ = 245.6 m*M*. The partition coefficients (P = $[compound]_{octanol}/[compound]_{water}$) for I and V were 7.26 and 87.6, respectively.

Anticancer Screening—On day 0, groups of 4–8 male and/or female mice (~25 g) were inoculated with cancerous cells (either L-1210 or P-388) at a level of either 10^5 or 10^6 cells/mouse. On the specified day (see Tables II and III), the test groups received single intraperitoneal injections of the test compound using a suitable vehicle and various doses; untreated control groups were injected with vehicle only. Each test compound was studied in parallel with I·H_2O, which served as a positive control for the screening protocol. Evaluations using mean survival times for test groups *versus* untreated control groups were scored as either increased life-span or test/control percentages. Pertinent experimental details and results are summarized in Tables II and III.

RESULTS AND DISCUSSION

Syntheses and Structure Determinations—Very little is known (25) about the *N*-fluorination of phosphoramides. The reaction of fluorine with the secondary phosphoramidic moiety, P(O)NHR, results in *N*-fluorination followed by P—N bond cleavage to give *N*-fluoroamines (25), whereas the secondary sulfonamide moiety SO_2NHR, reacts cleanly with trifluoromethylhypofluorite to give the corresponding *N*-fluorosulfonamide (26). Thus, it appeared that IV might be obtained directly from I using this hypofluorite reagent. Consideration of reported reaction mechanisms (26), in addition, suggested that subsequent P—N bond cleavage in IV might be minimized by using a fairly large molar excess of I, relative to the fluorinating reagent. Accordingly, the anhydrous reaction of I with 0.3 equivalents of the fluorinating agent led to ^1H-decoupled ^{31}P-NMR spectra at 40.25 and 121.5 MHz showing a singlet due to residual I (δ10.76) and a 1:1 doublet (J_{PF} = 22 Hz), which was shifted to lower field (δ12.64) and indicated the presence of IV. Following column chromatography, the structure of IV (20% yield) was unambiguously established by MS, ^{19}F-NMR, and ^{13}C-NMR. Comparison of ^{13}C-NMR parameters (Table I) for I, III, and IV showed that the electronegative OH and F substituents caused relatively large downfield shifts ($\Delta\delta$11–13) of the C-4 resonance absorption. This effect, although smaller ($\Delta\delta$1–2),

Table III—Anticancer Screening Data for 3-Chlorocyclophosphamide (V) Enantiomers and Racemic Cyclophosphamide Against P-388 Tumor Cells in Mice [a]

Compound [b]	Mouse Type	Injection Data [c]	Dose, mg/kg	ILS [b], %
(*R*)-V	BDF_1	10^6 cells/	113 [d]	31.6
(in vehicle 4)	(n = 8)	mouse	56	17.4
		on day 0	28	6.3
(*S*)-V			113 [d]	31.6
(in vehicle 4)			56	22.5
			28	9.3
(*R,S*)-I·H_2O			100 [d]	113.2 [e]
(concurrent positive			50	87.3
control)			25	47.8

[a] Conducted at Otsuka Pharmaceutical Co. Ltd., Osaka, Japan. The control groups (n = 23) received 5 ml/kg of the vehicle. [b] Vehicle 4 and ILS as defined on Table II. [c] Injected intraperitoneally; test compounds were given orally on day 2. [d] Doses of 113 mg/kg of (*R*)- or (*S*)-V and 100 mg/kg (*R,S*)-I·H_2O represent equimolar doses. [e] One 30-day survivor.

was evidenced also by the C-5 signal positions, whereas the more remote carbons (C-6, C-α, and C-β) in I, III, and IV were not significantly influenced by the nature of the N-3 substituent. The long-range $^{13}C—^{19}F$ couplings observed for C-4 (J = 11.6 Hz) and C-5 (J = 9.8 Hz) were also indicative of the location of the fluorine substituent in IV[13].

^{31}P-NMR was used to monitor the reaction of I·H_2O with 10 equivalents of sodium hypochlorite in 1:1 (v/v) chloroform–water, and it was found that I (δ12.10) underwent a gradual conversion ($t_{1/2} \cong$ 2 hr) to a single product having a downfield-shifted resonance signal (δ15.95). The ^{13}C-NMR parameters (Table I) measured for the isolated reaction product were in accord with V (87% yield), which gave rise to downfield-shifted absorptions for C-4 ($\Delta\delta$15) and C-5 ($\Delta\delta$3), in comparison with I[14]. Analogous reactions of sodium hypochlorite with 5,6-benzocyclophosphamide (VII) and *cis*- and *trans*-4-phenylcyclophosphamide (VIII and IX) gave 80, 59, and 94% yields, respectively, of the corresponding 3-chloro derivatives, X–XII. The substantial downfield shift of each compound's C-4 signal ($\Delta\delta$6 for X, $\Delta\delta$13 for XI and XII), relative to its precursor, was again characteristic of the assigned structure[15].

The conversion of the 4-phenyl analogues of I to XI and XII were ~5–10-times slower than chlorination of either I or VII. This rate difference was attributed to steric hindrance of the N-3 position by the adjacent 4-phenyl substituent, which is evident in the crystal structure of VIII (23).

Reaction of a methylene chloride solution of I·H_2O with 1.04 equivalents of bromine in the presence of potassium carbonate gave a quantitative yield of VI. As with IV and V, introduction of the 3-bromo substituent resulted in characteristic downfield shifts of the C-4 ($\Delta\delta$17) and C-5 ($\Delta\delta$4) ^{13}C-NMR signals, compared with I (Table I).

Reactivity Studies—Fluoro-derivative IV was indefinitely stable in chloroform solution at −80° and failed to undergo reductive dehalogenation to give I on contact with thiophenol in chloroform (15 hr, 25°). Compound IV was also stable for 4 hr at 25° in a 10:1 (v/v) mixture of 2,6-lutidine (pH 7.2) and *p*-dioxane. On the other hand, ^{31}P-NMR spectra in unbuffered water showed the gradual disappearance of IV (δ16.17) with concomitant formation of decomposition products giving rise to singlets at δ12.78, 12.53, and 1.30. The characteristic doublet for IV was no longer detectable after 16 hr, and the three product signals in the now acidic solution (pH 3) had relative intensities of 33, 50, and 17%, respectively. The absence of resolvable $^{19}F—^{31}P$ couplings in the products indicated the absence of P(O)NF moieties; the addition of potassium dihydrogenphosphate established that the signal at δ1.30 was due to inorganic phosphate, which most likely resulted from proton-catalyzed P—N bond hydrolysis (27, 28). Since the P—N bonds in I are known to be sensitive to acid-catalyzed hydrolysis (27), the aforementioned results were consistent with the decomposition of IV to I and hydrofluoric acid, which then afforded hydrolytic products of the type previously found with I (27). Solutions of IV or IV–bovine serum albumin (20:1) in 0.1*M* NaCl gave results similar to those obtained in unbuffered water.

Surprisingly, ^{31}P-NMR studies of the chloro derivative (V) revealed reactivity patterns opposite to those of IV: V was instantaneously (<3 min) reduced to I by 20-fold molar excesses of either thiophenol (in chloroform) or mercaptoethanol (in water) at 25°, but there was only 8% conversion of V to I in unbuffered water after 7 days at 25°. Analogous results were obtained with the bromo compound (VI) which was somewhat more reactive than V in water, *i.e.*, 8% hydrolysis after 16 hr at room temperature.

The marked susceptibility of V and VI to reductive generation of I *via* reaction with sulfhydryl-containing compounds led to the use of the fluoro derivative (IV) to assess the possibility of an enzyme-mediated reduction process formally analogous to that found for III and mouse liver microsomes (6). Due to the hydrolytic instability of IV, incubations were terminated after 10 min, during which time there was negligible hydrolysis to I. The 37° incubations of IV in saline were performed in parallel with III, which served as a control for establishing the reducing capability of the whole-liver slices. Chloroform extraction of the incubation mixtures containing 3.6 m*M* substrate and 1-g portions of liver slices afforded samples having ^{31}P-NMR spectra which showed 96:4 and 79:21 relative ratios of III–I and IV–I, respectively; however, sample weights and absolute signal intensities for III and IV revealed that, unlike III, ~60% of IV had not been extracted. Thus, the corrected yield of I from IV was ~8%, roughly twice the yield of I from III. Use of 3-g portions of sliced liver failed to significantly increase the yield of I from either III or IV; the recovery of unreacted IV was only ~20%, whereas unreacted III was again quantitatively recovered.

Anticancer Screening—The comparatively stable compounds, *i.e.*, IV, V, and XI, were screened for anticancer activity using established protocols for analogues of I. Unfortunately, however, it was not possible to use a single methodology for the evaluations, as three independent laboratories were involved in these tests. From the data summary given in Table II, it was evident that while IV was the most active analogue in the mouse L-1210 leukemia system, it was less effective than the parent prodrug, I.

The individual enantiomers of V had statistically identical therapeutic efficacy in both the L-1210 (Table II) and P-388 (Table III) screens. The absence of significant therapeutic differences between (*R*)- and (*S*)-V thus paralleled findings reported for the enantiomers of I (29). As found for I and V, the introduction of a 3-chloro substituent (XI) into the 4-phenylcyclophosphamide analogue (VIII) had a negative impact on the anticancer activity.

Partition Coefficients—Conversion of the N—H moiety in I to either N—F or N—Cl moieties must alter solubility characteristics which are related, in part, to the aforementioned anticancer screening results. The magnitude of the solubility change resulting from the *N*-chlorination of I was therefore quantified using ^{31}P-NMR to measure partition coefficients (P) in octanol–water, where $P = [\text{compound}]_{\text{octanol}}/[\text{compound}]_{\text{water}}$. By this method, log P = 0.86 for I[16] and 1.94 for the chloro-derivative V, revealing a 12-fold greater partitioning of V into octanol. The increased lipophilicity resulting from this introduction of chlorine [$\pi_{Cl} \equiv \log P_V - \log P_I = 1.08$] was comparable in magnitude to the influence of introducing a *para* chlorine substituent in phenol [π_{Cl} = 0.93 (31, 32)]. This correlation allowed the calculation of log P for IV, which was not studied by the ^{31}P-NMR method because of possible complications due to solvolysis. The *p*-fluorophenol π_F value of 0.31 (31) thus gave log $P \cong$ 1.22 for IV, indicating only an approximately twofold increase in lipophilicity, relative to I.

CONCLUSIONS

3-Chlorocyclophosphamide (V) was rapidly converted to cyclophosphamide (I) on contact with sulfhydryl-containing compounds and could thus afford the same level of anticancer activity as I, if the unmasking of V were fast, relative to the metabolic oxidative activation (2) of either V or I. On the other hand, the 12-fold increase in the lipophilicity of V, relative to I, might decrease its accessibility to sulfhydryl moieties and thereby account for the lower therapeutic efficacy found for V *versus* I. Sulfhydryl-mediated chemical reduction of the *N*-fluoro analogue IV was a very slow process, by comparison with V; however, IV was calculated to be considerably less lipophilic than V and, moreover, the possibility for *in vivo* enzymatic conversion of IV to I was supported by incubation experiments with mouse liver slices. Consequently, the anticancer activity of IV, although lower than that of I, was tentatively ascribed to its func-

[13] For directly bonded carbon-13 and fluorine-19 in analogous structures, $J \cong$ 175 Hz (17).

[14] The alternative 4-chlorocyclophosphamide structure was ruled out by obtaining the ^{13}C-NMR spectrum with off-resonance hydrogen decoupling, which demonstrated that C-4 was bonded to two protons.

[15] The possibility of benzylic chlorination of VIII to give its 4-chloro derivative was excluded by ^1H-NMR homonuclear decoupling, *i.e.*, simultaneous irradiation of the axial and equatorial C-5 hydrogens (δ2.0–2.3) in product XI led to the observation of the C-4 hydrogen (δ4.7) as a 1:1 doublet with $^3J_{HP}$ = 19 Hz.

[16] Hansch and Leo (30) have cited an unpublished log P value of 0.63 for I in octanol–water.

tioning as an enzyme-activated "pro-prodrug," which reductively released prodrug I for subsequent oxidative conversion of I to a known array of oncostatic metabolites (2). Alternatively, and by analogy to the metabolism of I (2), oxidation of the C-4 position in IV could afford the *N*-fluoro derivative of phosphoramide mustard as the ultimate lethal cytotoxic agent. However, the highly electronegative fluorine substituent would, as in *N*-hydroxyphosphoramide mustard (6), diminish the phosphoramide mustard's alkylating activity by several orders of magnitude, which argues against a mechanism of action for IV wherein the N—F bond remains intact.

For the extensions of the presently reported *N*-fluorination of I, candidate structures having secondary phosphoramide groups include the anticancer drug isophosphamide (8, 9) and various cytotoxic phosphoramidic nitrogen mustards (2). Moreover, there are numerous drugs of various types which contain secondary sulfonamide and, especially, carboxamide groups that offer the possibility for *N*-fluorination by trifluoromethylhypofluorite. It is hoped that the studies reported herein will stimulate an interest in *N*-fluorinated amido groups as a new approach for investigating prodrugs, "pro-prodrugs," and congeners.

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ACKNOWLEDGMENTS

This investigation was supported in part by Research Grant CA-21345 from the National Institutes of Health (to Gerald Zon).

The 100-MHz and 300-MHz NMR spectrometers were made available to Gerald Zon, as a Visiting Scientist, at the Food and Drug Administration, Bureau of Biologics. The authors thank Drs. Peter Roller and Richard Miller of the National Cancer Institute for obtaining the mass spectra.