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Solution Conformational Analysis, Crystal Structure Determination, and Anticancer Activity of Ring-Constrained 1,3,2-dioxa- and Oxazaphosphorinane Cyclophosphamide Analogues

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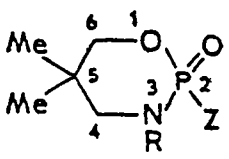
ABSTRACT Conformational analysis of 2-[bis-(2-chloroethyl)amino]-2-trans-tetramethylene-1,3,2-dioxa- and oxazaphosphorinanes *5-8*, cyclophosphamide-type anticancer drugs, show that diastereomeric six-membered ring 1,3,2-dioxaphosphor- inanes can adopt either a chair conformation with bis-(2-chloroethyl)amino mustard group equatorial (*5*) or twist-boat conformation with the bis-(2-chloroethyl)amino group pseudoequatorial (*6*). An X-ray crystal structure and NMR solution conformational analysis of the diastereomeric oxazaphosphorinanes *7* and *8* show that *7* adopts a chair conformation with equatorial bis-(2-chloroethyl)amino group, and *8* exists as mixture of chair-twist boat conformations with ca. 50% twist boat population. The diastereomeric pairs *5/6* and *7/8* show similar, moderate activity against lymphocytic leukemia P388 cells in mice. This is explained in terms of a flexible chair-twist boat interconversion.

INTRODUCTION

The study of 1,3,2-dioxa- and 1,3,2-oxaza-phosphorinanes is of current interest[1] with regard to the understanding of electronic and steric effects on the conformational properties of the phosphorinane six-membered ring. For example, Bentrude and coworkers[2] have recently reported a systematic conformational study of 5,5-dimethyl-2-oxo-2-Z-1,3,2-oxazaphosphorinanes:

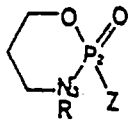
R = H, Z = Me_2N
R = H, Z = Et_2N
R = H, Z = $isoPr_2N$
R = H, Z = $(ClCH_2CH_2)_2N$



1, R=H; Z=$N(CH_2CH_2Cl)_2$

2, R=CH_2CH_2Cl; Z=$N(CH_2CH_2Cl)_2$

3, R=CH_2CH_2Cl; Z=$NHCH_2CH_2Cl$

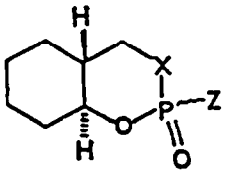


Cyclophosphamide *1*, trophosphamide *2*, and isophosphamide *3*, which all contain the 1,3,2-oxazaphosphorinane ring, are important anticancer drugs.[3,4]

Although many analogues of *1* have been synthesized, none has been found to be more active than the parent compound.[3,4] It is now believed[5,6] that the metabolism of cyclophosphamide involves an initial enzymatic hydroxylation at the C-4 position to form the active metabolite, 2-[bis-(2-chloroethylamino)]-4-hydroxytetrahydro-2H-1,3,2-oxazaphosphorinane-2-oxide, *4*. The selective cytotoxic effect of *4* towards neoplastic cells, has been suggested[6] to be derived from differences in the toxification and detoxification processes. Toxification has been suggested[7] to proceed through a chemical β-elimination that requires at least one hydrogen at the C-5 position in *1*. A study[8] showed that a cyclophosphamide derivative substituted at C-4 with two methyl groups possessed moderate drug activity, thus raising a question as to the current accepted mechanism of activation.

In this paper we report the preparation of diastereomeric pairs of the cyclophosphamide analogues, 2-[bis-(2-chloroethyl)amino]-2-trans-tetramethylene-1,3,2-dioxa- and oxazaphosphorinane *5-8* and studies of their conformation by ^1H, ^{13}C, ^{31}P NMR, and IR spectroscopy techniques.

5, *6*, X = O; Z=$N(CH_2CH_2Cl)_2$
7, *8*, X = NH; Z=$N(CH_2CH_2Cl)_2$
9, X = O; Z=OPh



An X-ray analysis of *7* was also performed, revealing that *7* exists in a flattened chair conformation with a pseudoequatorial N-mustard group. Finally, the anticancer activities of *5-8* were examined and to our knowledge this is one of the few examples of a comparison of the biological activity of both the oxaza- and dioxaphosphorinane ring system in cyclophosphamide type drugs.[7,8]

EXPERIMENTAL SECTION

Methods and Materials

^1H and ^{31}P NMR were recorded on a Bruker WP-200 spectrometer at 200 and 80.1 MHz, respectively, or proton NMR on a 360 MHz Nicolet or 60 MHz Varian A-60 or EM-360 spectrometers. Chemical shifts in parts per million for ^1H and ^{13}C NMR spectra are referenced to internal Me_4Si and for ^{31}P NMR are referenced to external 85% H_3PO_4. Infrared spectra were obtained on an AEI MS-30 spectrometer. Melting points were taken on a Thomas-Hoover apparatus and are uncorrected.

Chemicals were generally of highest purity. Baker analyzed 60-200 mesh silical gel was used for chromatography after being activated at 130°C overnight. Triethylamine, phosphoryl chloride, and methylene chloride were distilled before use.

X-ray Crystal Structure of 7

The crystals of 7 were triclinic, space group P 1, a = 5.559(2), b = 9.434(3), c = 14.215(3) Å, α = 95.80(2)° β = 94.10(3)°, γ = 90.61(3)°, V= 739.6(4) Å^3, Z = 2, D_c = 1.415 g cm^{-3}.

Intensity data were collected on a Picker FACS-1 automatic diffractometer modified with the Krisel Control update package. A total of 1795 unique reflections in the range $2.0 < 2\theta < 15.0°$ (of type ± h, k, l) with $F > 2.0\sigma(F)$ were used in the solution of the structure via direct methods (ACSHEL-X, G. Sheldrick, Programs for Crystal Structure Determination, Cambridge, 1975). The data were corrected for absorption. The structure was refined to R = .0727, and R_w = 0.0647 using 178 variable parameters and the 906 data between 2θ = 0° and 2θ = 35°. In the final model H atoms were inserted in calculated positions and anisotropic thermal parameters used for all non-hydrogen atoms.

SYNTHESES

[Bis-(2-chloroethyl)amino] phosphoryl dichloride. To a magnetically stirred solution of phosphoryl oxychloride (2.6 g, 0.01 mol) in 50 mL of methylene chloride at -5° was added dropwise a solution of bis(2-chloroethyl)amine hydrochloride (0.01 mol) and triethylamine (0.02 mol) in 50 mL of methylene dichloride over 1 h. The reaction mixture was stirred overnight at room temperature, concentrated in vacuo and mixed with bensene. The precipitated amine salt was filtered off and the filtrate was crystallized in ethyl acetate/hexane to give light yellow crystals, m.p. 46-48°. (lit[32] 46°) ^{31}P NMR ($CDCl_3$): 17.5 ppm; ^{13}C NMR (DMSO-d_6): 39.17, 42.07.

trans-2-Cyanocyclohexanol. To a magnetically stirred solution of 7.0 g of sodium cyanide in 60 mL of acetonitrile and 200 mL of water, 5.0 g of cyclohexene oxide was added with catalytic amount of 12-crown-4. The two phase reaction mixture was stirred for 40 h at 45°C. The unreacted sodium cyanide was filtered off, and the filtrate was concentrated in vacuo. The residue was then extracted with chloroform (3x100 mL). The combined organic layers were dried over anhydrous magnesium sulfate, concentrated in vacuo to give a light yellow oil, which was crystallized in ethyl acetate and hexane with cooling. m.p. 42° (lit[33]: 46°). ^1H NMR ($CDCl_3$) δ 1.0-2.40 (m, 9H, ring hydrogens), 2.50-3.05 (m, 1H, OCH), 3.18 (s, 1H, -OH); ^{13}C NMR ($CDCl_3$) ~23.45, 23.95, 28.15, 33.81, 37.52, 70.53, 123.56; IR (KBr): 3470, 2960, 2950, 2870, 2260, 1450, 1325, 1175, 1125, 1080, 1012 cm^{-1}.

trans-2-(Aminomethyl)cyclohexanol. To a magnetically stirred suspension of 3.75 g of lithium aluminum hydride in 150 mL of anhydrous ether, 4.0 g of trans 2-cyano cyclohexanol was added dropwise at room temperature over 40 min. The reaction mixture was then gently boiled for 4 h. The mixture was worked up by successively adding 4 mL of water, 4 mL of 15% KOH and 12 mL of water. The white inorganic salt was filtered off and the filtrate was extracted with ether, concentrated and dried over anhydrous magnesium sulfate to give a light brown liquid. This was vacuum dried and used without further purification. ^1H NMR[33] (CCl_4)δ 0.6-2.10 (m, 9H, ring hydrogens), 2.3-3.60 (m, 3H, -OCH and NCH_2), 3.0 (s, 3H, -OH and -NH_2); ^{13}C NMR ($CDCl_3$) 22.54, 25.41, 28.91, 35.02, 45.33, 47.88, 76.50.

trans-2-Hydroxycyclohexane carboxylic acid. trans-2-Cyanocyclohexanol (4.6 g) was hydrolysed in alcoholic KOH (10 g KOH in 100 mL of 95% ethanol) under reflux overnight. The reaction mixture was carefully acidified with cold 6M HCl to pH 2 with cooling in ice. The acidified solution was then extracted with $CHCl_3$ (200 mL x2). The combined organic layer was dried over anhydrous magnesium sulfate and concentrated in vacuo to give 3.0 g of trans 2-hydroxy-cyclohexane carboxylic acid (60%); ^1H NMR ($CDCl_3$) δ 0.8-2.35 (m, 9H, ring hydrogens), 2.75-4.20 (m, 2H, -CHOH), 6.3-7.2 (br s, 1H, COOH).

trans-2-(Hydroxymethyl)cyclohexanol. Trans-2-Hydroxycyclohexane (2.5 g) carboxylic acid in 50 mL of anhydrous ether was added over 30 min to a stirred mixture of lithium aluminum hydride (2.0 g, 0.051 mol) in 50 mL of ether. The reaction mixture was left to stir overnight and heated under reflux for 2 h.[34] This was followed by successive addition of 2 mL of water, 2 mL of 15% KOH and 6 mL of water. The granular precipitate was filtered off and the residue was extracted with ether and concentrated in vacuo to give a light brown liquid (1.4 g, 62%). This was distilled to give a colorless viscous liquid, b.p. 98-100°, 0.8 mm (lit[35] b.p.: 101-103°, 0.96 mm). ^1H NMR ($CDCl_3$) δ 0.6-2.38, (m, 9H, ring hydrogens), 3.32-4.0 (m, 3H, OCH, and OCH_2), 4.40 (s, 2H, -OH).

2-[Bis-(2-chloroethyl)amino]-2-ozo-trans-5,6-tetramethylene-1,3,2-diozaphosphorinane, 5 and 6. To 2.6 g (0.01 mol) of bis(2-chloroethyl)amino phosphoryl dichloride in 100 mL of THF, 1.3 g (0.01 mol) of trans 2-hydroxylmethyl-cyclohexanol and 2.3 mL (0.02 mol) of triethylamine in 50 mL of THF was added dropwise over 1 h with vigorous stirring. The reaction was left to stir overnight and heated at reflux for 6 h under nitrogen. After cooling, the amino salt was filtered off and filtrate was concentrated in vacuo to give a light yellow oil. ^{31}P NMR showed mainly two peaks at 6.7 ppm and 2.7 ppm in a ratio of 1.6:1 if THF is used as the solvent, 1.3:1 if ethyl acetate or ether is used. The crude product of two isomers was separated by flash chromatography on a 200/400 mesh 'Aldrich Davisil' silica gel using chloroform/methanol (99:1) as eluent. The fast-eluting isomer 5 was crystallised from ethyl acetate/hexane to give pure granular crystals (m.p. 81.5-82.5°) and the slow-eluting isomer 6 give colorless fine needle crystals (m.p. 62-64°). isomer 5: 6.70 ppm, slow isomer 6: 2.70 ppm. ^1H NMR ($CDCl_3$) 5: δ 0.80-1.02 9m, 1H), 1.20-1.35(m, 2H), 1.35-1.50 (m, 1H), 1.60-1.70 (m, 1H), 1.70-1.90 (m, 3H), 3.32-3.48 (m, 4H, NCH_2), 3.61 (t, J= 7.0 Hz), 4.11 (dddd, J=11.0 Hz, J=4.5 Hz, J= 22.8 Hz), 4.21 (d of t, J=11.0 Hz, J=11.0 Hz, J= 1.5 Hz), 4.28 (J= 11.0 Hz, J= 10.4 Hz,

J=4.1 Hz). *6*: 1.0-1.21 (m, 1H), 1.4-1.55 (m, 2H), 1.7-1.87 (m, 1H), 1.88-1.98 (m, 2H), 2.0-2.2 (m, 1H), 2.24-2.35 (m, 1H), 2.35-2.52 (m, 1H), 3.57 (t, J=6.84 Hz, 2H, NCH_2), 3.61 (t, J=6.84 Hz, 4H, $ClCH_2$), 4.15 (ddd, J= 9.4 Hz, J= 10.4 Hz, J= 11.4 Hz), 4.24 (d of t, J= 10.6 Hz, J= 10.6 Hz, J = 4.3 Hz, J= 2.2 Hz), 4.50 (ddd, J= 11.2 Hz, J= 10.4 Hz, J= 5.4 Hz). ^{13}C NMR ($CDCl_3$), *5*: 24.04, 24.54, 25.50, 32.84 (d, J=8.24 Hz), 41.40 (d, 5.63 Hz), 42.43, 49.54 (d, 4.22 Hz), 71.29 (d, 5.63 Hz), 80.9 (d, 4.22 Hz). *6*: 23.87, 24.17, 26.43, 32.91 (d, 6.03 Hz), 40.01 (d, 11.86 Hz), 41.83, 49.17 (d, 4.02 Hz), 72.26 (d, 6.03 Hz), 82.56 (d, 7.24 Hz); IR ($CDCl_3$) *5*: 3150 (w), 2940 (m), 2850 (w), 2250 (s), 1450 (m), 1475 (m), 1240 (s), 1090 (s), 1080 (s), 1020 (s), 1020 (s), 980 (s), 960 (s), 960 (s), 900 (s). *6*: 3050 (w), 2940 (w), 2250 (s), 1685 (w), 1450 (m), 1380 (m), 1240 (w), 1210 (w), 1080 (m), 790 (s); MS, *5*: m/e 316 (.5), 266.1 (51.7), 174.1 (30.7), 172.1 (100), 118.0 (45.8), 95.1 (23.2),, 67.1 (36.5), 63.0 (22.0), 56.1 (18.3), 55.1 (28.8), 29.1 (19.7), 28.1 (20.8), 27.1 (39.8). *6*: 268.2 (25.5), 266.2 (73.8), 174.1 (32.4), 172.0 (100), 110.0 (36.5), 95.1 (25.0), 67.1 (27.0), 63.0 (17.9), 55.1 (21.2), 41.0 (30.0), 28.0 (19.5).

Anal. Calcd. for $C_{11}H_{20}NO_3PCl_2$: C, 41.77; H, 6.33; N, 4.43; P, 9.81. Found for *5*: C, 41.82, H, 6.42; N, 4.26; P, 10.11. *6*: C, 41.75; H, 6.32; N, 4.16; P, 10.15.

2-[Bis-(2-chloroethyl)amino]-2-oxo-trans-5,6-tetramethylene-1,3,2-oxazaphosphorinane 7 and 8

To a stirred solution of 2.34 mL (22.0 mmol) of triethylamine and 2.85 g (11 mmol) of bis(2-chloroethyl)amino phosphoryl dichloride in 100 mL of dry THF, 1.41 g (11 mmol) of trans-2-aminomethyl cyclohexanol in 50 mL of THF was added dropwise over 30 min at 0-5° under a nitrogen atmosphere. The reaction mixture was then left to stir overnight at room temperature. The amine salt was filtered off and the filtrate was concentrated in vacuo to give a light yellow oil. ^{31}P NMR of the crude product in $CDCl_3$ showed only two peaks at 12.9 ppm and 10.9 ppm in a ratio of 1.1:1. The crude product of two isomers was separated by flash chromatography on a 200/400 mesh "Aldrich Davisil" silica gel (1.25in. x 20in.) using chloroform/methanol (97:3) as eluent. The fast-eluting isomer was crystallized from chloroform/hexane to give colorless needle crystals (m.p. 120-121°) and the slow-eluting isomer give pure granular crystals (m.p. 96-98°). ^{31}P NMR ($CDCl_3$) of the fast isomer *7*, 12.9 ppm, slow isomer *8*, 10.9 ppm. ^1H NMR ($CDCl_3$, 360 MHz) *6 7*: 0.85-1.08 (m, 1H), 1.15-1.30 (m, 2H), 1.30-1.45 (m, 1H), 1.50-1.85 (m, 4H), 1.95- 2.10 (m, 1H), 2.45 (s, NH), 3.15 (m, 1H, H_2), 3.20 (m, 1H, H_1), 3.31-3.57 (m, 4H, NCH_2), 3.62 (t, J= 6.9 Hz, 4H, $-CH_2Cl$), 4.24 (d of t, J=10.6 Hz, J=10.6 Hz, J=4.3 Hz, J=2.2 Hz, 1H, H_6). *8*: 0.85-1.0 9m, 1H), 1.18 (m, 2H), 1.48-1.60 (m, 1H), 1.62-1.95) (m, 4H), 1.98-2.16 (m, 1H), 2.84 (m, -NH), 3.13 (dddd, J=21.4 Hz, J=6.8 Hz, J=4.5 Hz, 1H, H_1), 3.30-3.42 (m, 4H, $-NCH_2$), 3.45 (m, 1H, H_2), 3.67 (t, J= 6.84 Hz, 4H, $-CH_2Cl$). ^{13}C NMR ($CDCl_3$) *7*: 24.19, 24.76, 28.07, 33.30, (d, J=9.65 Hz), 40.66 (d, J= 2 Hz), 42.52, 47.94 (d, J= 3.0 Hz), 49.07 (d, J=4.02 Hz), 80.57 (d, J=5.83 Hz). *8*: 24.29, 24.75, 28.07, 33.35 (d, J= 7.64 Hz), 41.81 (d, J=9.65 Hz), 42.05, 47.19, 48.78 (d, J= 3.22), 83.0 (d, J=6.43 Hz). IR ($CDCl_3$) *7*: 3150 (w), 2930 (w), 2250 (s), 1790 (w), 1460 (m), 1280 (m), 1215 (m), 1090 (m), 790 (s). *8*: 3050 (w), 2930 (m), 2250 (m), 1450 (m), 1370 (m), 1250 (m), 1080 (m), 1000 (m), 895 (m). MS *7*: m/e 315.0 (.22), 267.2 (31.3), 265.2 (100), 174.1 (27.3), 110.2 (22.2), 94.1 (20.8), 93.1 (19.3), 92.1 (50.0), 81.1 (22.7). 67.1 (35.3), 30.1 (22.0), 28.0 (23.4). *8*: 315.1 (.65) 267.2 (20.1), 265.2 (65.5), 229.2 (26.3), 197.1 (37.7), 174.1 (92.4), 110.2 (31.7), 95.1 (18.3), 93.1 (38.1), 92.1 (55.7), 81.1 (34.6), 79.1 (26.1), 67.1 (58.2), 65.1 (28.5), 56.1 (54.5), 55.1 (32.1), 54.1 (35.4), 49.1 (30.7), 42.1 (71.5), 41.1 (100), 30.1 (36.1), 29.1 (54.5), 27.1 (69.2).

Anal. Calcd for $C_{11}H_{21}N_2O_3PCl_2$: C, 41.90, H, 6.66; N, 8.88. Found for *7*: C, 41.82; H, 6.52; N, 8.85. *8*: C, 41.92; H, 6.51; N, 8.95.

RESULTS

Syntheses

The bicyclophosphamides *5-8* were prepared from [bis-(2-chloroethyl)-amino] phosphoryl dichloride and the appropriate diol or amino alcohol. Cyclohexene oxide was used to insure the required trans stereochemistry for the diol and amino alcohol. Diastereomeric mixtures of *5/6* were obtained in a ratio of 1.6:1, and *7/8* in a ratio of 1.1:1 if THF was used as solvent. For convenience, based upon the stereochemical relationship between the $-N(CH_2CH_2Cl)_2$ group and C-5 cyclohexane ring carbon, *6* and *8* are defined as the "trans" isomers, and *5* and *7* as the "cis" isomers.

X-ray Structure

An ORTEP perspective drawing of *7*, along with the labeling scheme appears in Figure 1. Atomic coordinates

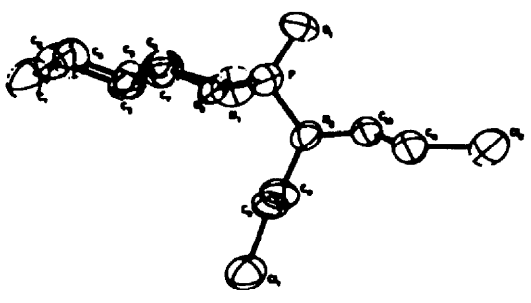


Figure 1 ORTEP Plot of 7. Hydrogen atoms have been omitted for clarity.

Table I. Final Positional parameters ($X10^4$) for Non-Hydrogen atoms in phosphorinane 7[a]

ATOM	10^4X	10^4Y	10^4Z
C(1)	-0564(31)	2776(15)	4592(10)
C(2)	0281(27)	3016(14)	3617(10)
C(3)	0772(25)	1615(14)	3027(10)
C(4)	-1349(24)	0653(13)	2937(10)
C(5)	-2223(27)	0381(14)	3878(9)
C(6)	-2737(30)	1757(16)	4470(12)
C(7)	1638(24)	1904(13)	2080(10)
N(1)	1888(20)	0611(12)	1460(9)
P	0030(7)	-0783(4)	1399(3)
O(1)	-1896(14)	-0899(7)	0638(5)
O(2)	-0776(14)	-0692(8)	2445(5)
N(2)	1596(19)	-2284(8)	1318(6)
C(8)	3586(24)	-2445(13)	2051(9)
C(9)	2797(23)	-3530(15)	2670(9)
Cl(1)	5278(7)	-3882(4)	3492(2)
C(10)	1468(22)	-3258(11)	0473(7)
C(11)	3364 (21)	-2866(11)	-0181(7)
Cl(2)	3369 (7)	-4190(3)	-1162(2)

a. Numbers in parentheses are estimated standard deviations. Atoms are labeled to agree with Figure 1.

Table II. Selected Bond lengths (Å) and Bond Angles (deg) for 7.

Bond Lengths					
P-O(1)	1.461	(7)	P-O(2)	1.578	(7)
P-N(1)	1.655	(11)	P-N(2)	1.668	(9)
N(2)-C(8)	1.485	(12)	N(2)-C(10)	1.433	(11)
C(8)-C(9)	1.497	(13)	C(10)-C(11)	1.521	(13)
C(9)-Cl(1)	1.799	(11)	C(11)-Cl(2)	1.775	(10)
N(1)-C(7)	1.444	(13)	C(3)-C(7)	1.511	(14)
C(3)-C(4)	1.471	(14)	C(4)-O(2)	1.436	(11)
C(2)-C(3)	1.531	(13)	C(1)-C(2)	1.531	(15)
C(1)-C(6)	1.524	(16)	C(4)-C(5)	1.499	(14)
Bond Angles					
O(1)-P-O(2)	116.5	(5)	O(1)-P-N(1)	117.0	(5)
O(1)-P-N(1)	108.3	(5)	O(2)-P-N(1)	101.5	(5)
O(2)-P-N(2)	102.6	(5)	N(1)-P-N(2)	109.9	(6)
P-N(2)-C(8)	118.1	(8)	P-N(2)-C(10)	122.3	(8)
C(8)-N(2)-C(10)	118.1	(9)	P-O(2)-C(4)	120.7	(7)
P-N(1)-C(7)	123.9	(7)	N(1)-C(7)-C(3)	112.3	(11)
O(2)-C(4)-C(3)	109.8	(10)	C(7)-C(3)-C(4)	112.9	(11)
C(1)-C(2)-C(3)	112.2	(11)	C(2)-C(1)-C(6)	109.6	(13)
C(6)-C(5)-C(4)	111.7	(12)	C(7)-C(3)-C(2)	110.4	(11)

Table III. Selected Torsional Angles of Phosphorinane 7[a]

C6-C1-C2-C3	-54.1(6)	C1-C2-C3-C4	+54.9(6)
C2-C3-C4-C5	-54.8(6)	C3-C4-C5-C6	+55.1(6)
C4-C5-C6-C1	-54.6(6)	C5-C6-C1-C2	+53.9(6)
C7-C3-C4-O2	+59.9(6)	C3-C4-O2-P	-60.7(5)
C4-O2-P-N1	+42.3(6)	O2-P-N1-C7	-31.0(5)
P-N1-C7-C3	+37.1(5)	N1-C7-C3-C4	-49.0(6)
C4-O2-P-O1	-85.9(5)	C4-O2-P-N2	+156.0(5)
O1-P-N1-C7	+97.1(6)	N2-P-N1-C7	-139.0(6)
O1-P-N2-C10	+17.8	O1-P-N2-C8	-176.8

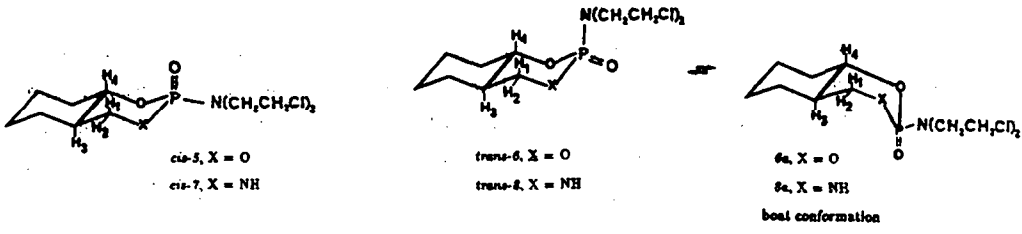
a. Numbers in parentheses are estimated standard deviations; atoms are labeled to agree with Figure 1.

for non-hydrogen atoms appear in Table I, and important bond lengths and angles are in Table II. The 1,3,2-oxazaphosphorinane ring system of *7* is in the chair conformation with the [bis-(2-chloroethyl)amino] group in the pseudo-equatorial position and the phosphoryl oxygen in the pseudo-axial position. Table III gives the ring torsional angles, which also show the heterocyclic ring to be quite chairlike but with considerable flattening at the phosphorus end.

The geometry about the [bis-(2-chloroethyl)amino] nitrogen atom, N(2), is nearly planar, the sum of bond angles about N(2) being 358.5° compared to the tetrahedral sum of 328.4° and the planar sum of 360°. The geometry about the ring nitrogen, N(1) is pyrimidal [with a sum of bond angles around N(1), 331.2°] and a typical bond length 1.655 (.011) Å.

Proton NMR Parameters and Solution Configurational and Conformational Analysis of *5* and *6*.

Chemical shifts and coupling constants for the protons of dioxaphosphorinanes *5* and *6* were derived essentially from the first-order ^{1}H NMR spectra obtained at high field (360 MHz) and are listed in Tables IV and V. Vicinal three-bond coupling constants generally follow a Karplus-type relationship, in which the coupling constant varies as a function of the torsional angle. This three-bond proton-phosphorus coupling has been widely used in determining the conformation of 1,3,2-dioxa- and oxazaphosphorinane systems.[1a,2,9-11] The dioxaphosphorinane ring in diastereomer *5* appears to be entirely in a chair conformation based on a comparison of the large $^3J_{2P}$ coupling constant, 22.8 Hz, and a small $^3J_{1P}$ value, 4.1 Hz, with similar coupling constants in the structurally related compound dioxaphosphorinane ester *9a* (Table IV), which is also believed to be almost entirely in the chair conformation ($^3J_{2P}$, 24.4 Hz).[10]



Scheme I

Diastereomer *6* appears in solution to be almost entirely in a boat or time-averaged twist-boat conformation *6a* because of its much smaller $^3J_{2P}$ coupling constant, 11.2 Hz, and larger $^3J_{1P}$ coupling constants, 11.4 Hz. This result is similar to that in our study[10] of 2-aryloxy-2-oxo-dioxaphosphorinanes *9b*. These ^{1}H NMR results combined with the ^{31}P, ^{13}C NMR and IR spectral data discussed below, generally support the assignment of *5* as the "cis" diastereomer with the bis-(2-chloroethyl)amino group in the equatorial position of a chair dioxaphosphorinane ring and *6* as the "trans" diastereomer with the bis-(2-chloroethyl)amino group in a pseudoequatorial position of a twist-type dioxaphosphorinane ring (*6a*).

Support for these stereochemical assignments is provided by the ^{1}H chemical shifts of H-1, H-2 and H-4. When H-1 is cis to the P=O group, it is downfield of the diastereotopic H-2 and the H-4 protons. The opposite is true when H-1 is trans to the P=O group, as observed in both 1,3,2-dioxa- and oxazaphosphorinanes esters and may be a result of the deshielding effect of the P=O group.[11,19a] Furthermore, this deshielding effect is expected to be large when the P=O has a syn-axial relationship to the hydrogens, and small when the P=O has a syn-equatorial relationship to the hydrogen. Apparently cis-*5* adopts a conformation in solution with axial P=O group, and as a result there is a deshielding of the axial H-1 and H-4 protons in *5* relative to the equatorial H-2 proton (Table V).

Proton NMR Parameters and Configurational and Conformational Analysis of Diastereomers *7* and *8*.

The configuration of *7* and *8* have been established by the X-ray crystal structure determination of one of the diastereomers, *cis-7* (and confirmed by IR and NMR analysis). As discussed above for *5*/*6* the large $^3J_{2P}$, 21.4 Hz (Table IV), and small $^3J_{1P}$, 4.2 Hz, confirm that *7* exists largely in a chair conformation.

The conformation of trans-*8* has been analyzed by both ^{1}H and ^{13}C NMR. A moderately large $^3J_{2P}$ coupling constant of 17.5 Hs is observed for *8*, and this suggests that *8* exists in solution as a mixture of chair and twist-boat conformations, *8a*. A rough estimate of the equilibrium between chair-form *8* and twist-boat form *8a* can be made from a consideration of the population-weighted averaged coupling constants. Since *5* and *6* are almost entirely in

chair and twist-boat conformations respectively, the values of J_{2P}, 22.8 and 11.2 Hz for *5* and *6*, provide reasonable estimates for the limiting J_{2P}'s of pure chair and twist-type conformations, respectively. Using these values and the J_{2P} value of 17.5 Hz for *8*, we estimate that *8* exists as ca. 50/50 mixture of twist-boat/chair conformations in a 1% solution in $CDCl_3$. Unexpectedly, the H-1 chemical shift of *7* is upfield of the H-2 signal. This is not consistent with the expected deshielding of an axial P=O group, as discussed above for *5*.

^{13}C NMR Spectra

The conformations for *5-8* may also be deduced from the vicinal P-C three-bond coupling constants (Table VI) derived from the ^{13}C NMR spectra. In an earlier study by Gorenstein et al.[10], for *9* (R= various aryl esters), vicinal P-C coupling constants $^3J_{C-5P}$ in the range 8.9 - 9.9 Hz were used to assign a chair conformation, those about 6.1 Hz a twist boat, and in the range 7.8 - 8.5 Hz for a rapid equilibration between nearly equal populations of chair and twist boat. However, in the earlier work, the magnitude of the vicinal coupling apparently depended on the identity of the substituent group in addition to the torsional angle change. Thus, *5* and *7*, which are both in chair conformations, have $^3J_{C-5P}$ of 8.24 and 9.65 Hz respectively. A $^3J_{C-5P}$ = 6.03 Hz for *6* is consistent with the assignment of a twist boat conformation (this is also seen the large $^3J_{C-3P}$ = 11.86 Hz, indicating flipping at phosphorus with an almost 0° dihedral angle for P-N-C_7-C_3 or P-O-C_4-C_3.) Values for $^3J_{C-5P}$ of 7.64 Hz, and $^3J_{C-3P}$ of 9.6 Hz are consistent with the notion that *8* exists as a mixture of chair and twist-boat conformations.

Infrared Studies

Hydrogen bonding does not appear to play a major role[2] in the determination of the conformation of *7* or *8*, as no significant hydrogen-bonded NH adsorption at 3185-3230 cm^{-1} was observed. The use of the P=O bond stretching frequency in IR has been used to help establish the configuration of 1,3,2-dioxa- and oxazaphosphorinane[14,15] in many instances. Generally, in closely related phosphorinanes, an equatorial P=O group has a phosphoryl stretch ca. 20-30 cm^{-1} larger than an axial P=O group[16,17]. However, this correlation must be cautiously applied[2] and conclusions from P=O stretching frequencies are best when buttressed by results from other techniques such as ^1H, ^{13}C, ^{31}P NMR and X-ray analysis. Nevertheless, good conclusions can generally be reached if the phosphorinanes under study are very much alike, as in a comparison of diastereomeric pairs, such as *5/6* and *7/8*. The phosphoryl stretching frequencies in the IR spectra for 5-8 in different media are collected in Table VII.

In the solid state in KBr, *5* and *6* have similar P=O stretching frequencies, 1254 and 1266 cm^{-1}, respectively. This is consistent with a chair conformation for *5* with an axial P=O bond, and a twist-boat conformation for *6* with a pseudo-axial P=O bond. In solution, the P=O stretching frequencies for *5/6* are also quite comparable, confirming these assignments. Phosphorinane *7* has a lower stretching frequency, 1228 cm^{-1}, compared to that of *8*, 1245 cm^{-1}, which is also consistent with the assignments, i.e. axial P=O bond for chair conformation cis-*7* and equatorial P=O bond for a chair conformation trans-*8*. In $CDCl_3$ solution, in which apparently both chair and twist-boat conformations are observed for *8*, two P=O stretching frequencies are noted (1210 cm^{-1} for twist-boat *8a* and 1250 cm^{-1} for chair *8*). However, the P=O stretch for *7* in CCl_4 cannot readily be rationalized.

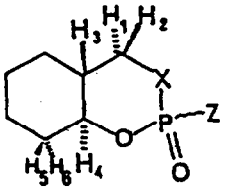
^{31}P NMR Spectra

In diastereomeric 2-oxo-1,3,2-dioxaphosphorinane esters, an upfield chemical shift in ^{31}P NMR will generally be observed for the 2-substitutent ester or amide group in an axial position compared to the equatorially substituted diastereomer.[1a,10,19,20] This effect is seen in the oxaza ester system as well.[19e] Diastereomeric pairs *5/6* and *7/8* follow this criterion (Table V), with the ^{31}P chemical shift of *5* 5.0 ppm upfield of *6* and *7* 2.6 ppm upfield of *8*. However, this ^{31}P correlation is not consistent with the solution twist-boat conformations for *6* and *8*. Thus, while the bis(-2-chloroethylamino) groups are axial in chair conformations for *6* and *8*, the substituent is pseudoequatorial in a twist boat conformation. In both chair *5* and *7* and twist-boat *6* and *8*, the bis(-2-chloroethylamino) group is in the same relative position and the ^{31}P chemical shifts of the diastereomers are expected to be similar.[1]

Anticancer Activity

The acute toxicity of the cyclophosphamide analogs[5-8], were evaluated using Swiss mice. At doses of 50, 100, 200, and 400 mg/kg of body weight of the Swiss mice, all animals remained alive indicating no acute toxicity of compounds 5-8 at the tested concentrations. Furthermore, no chronic toxicity was observed during the testing of compounds *5-8* against the lymphocytic leukemia p388 in CD_2F_1 male mice at 75 mg/kg/day, administered for 9 consecutive days. The decreased toxicity of compounds *5-8* as compared to cyclophosphamide might be attributed

Table IV. Selected ^{1}H NMR Spectral Parameters for 5–8



5, *6*, X = O; Z=N(CH$_2$CH$_2$Cl)$_2$

7, *8*, X = NH; Z=N(CH$_2$CH$_2$Cl)$_2$

9, X = O; Z=OPh

Coupling Constants

compd	J_{12}	J_{13}	J_{23}	J_1P	J_2P	J_4P	J_{34}	J_{45}	J_{48}
5	11.0	10.4	4.5	4.1	22.8	0	11.0	11.0	1.5
6	10.4	9.4	5.4	11.4	11.2	2.2	10.6	10.6	4.3
7	12.2	10.5	4.5	4.2	21.4	1.4	11.0	11.0	5.4
8	-	-	-	-	17.5*	1.1	11.0	11.0	4.3
9a	10.8	11.1	4.4	0.0	24.4	1.0	0		
9b	10.7	11.0	5.0	5.5	18.5	2.0			

*Obtained from coupled phosphorus spectrum.

Table V. Selected Chemical Shifts for Phosphorinanes 5-8.

Compd.	δH-1	δH-2	δH-4	δ^{31}P
5	4.28	4.11	4.21	6.7
6	4.50	4.15	4.24	2.7
7	2.84	3.15	3.93	12.9
8	3.20	3.15	4.14	10.3

to the fact that in the case of compounds *5-8* no formation of the very toxic acrolein during the metabolism of these compounds can be expected, whereas the metabolism of cyclophosphamide[18,21] is known to produce acrolein which is the source of severe side effects of the cyclophosphamide therapy.[22]

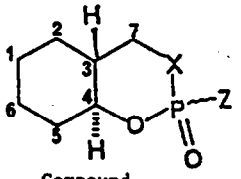
The anticancer activity of compounds *5-8* against p388 was found to be low to moderate depending on the concentration as evidenced by the increase in life span ranging between 37%-67% (Table VIII). No optimum dose response was attempted because of unrealistically high dose requirements to achieve this goal. The diester monoamide compounds *5* and *6* seem to be marginally less active than the monoester diamide derivatives *7* and *8*. However, the differences are considered to be within the experimental error. All compounds *5-8* are less active than the clinically used cyclophosphamide at comparable doses. Thus, the increase in life span for cyclophosphamide at 65 mg/kg/day was found[6] to be 239%.

DISCUSSION

X-ray Crystal Structure of 7.

When not sterically restricted, dialkylamino R_2N and P=O prefer a geometry with trigonal planarity about nitrogen and coplanarity between the P=O and R groups.[24,25] This usually is accompanied by a shortening of the P-N bond as a result of π bonding involving the nitrogen (or oxygen) p-orbital with either a phosphorus d-orbital[26] or a phosphorus substituent σ-orbital.[24b,27] Similar considerations hold for 2-oxo-2-alkylamino-1,3,2-dioxa- and oxazaphosphorinanes.[24,25] However, in six-membered dioxa- and oxazaphosphorinane ring systems with axial dialkyl amino groups (such as the N-mustard group), coplanarity between the P=O bond and exocyclic nitrogen C(10) and C(8) is not favored for this would result in non-bonded interaction of a sterically bulky β-chloroethyl group and the syn-axial hydrogens. This steric interaction is less important for an equatorial bis-(2-chloroethyl)amino group. Indeed, as seen in the crystal structure of *7*, the P=O and dialkylamino group are essentially coplanar. The O1-P-N2-C10 and O1-P-N2-C8 dihedral angles are 17.8° and -176.8°, respectively (Table III). The steric interaction of an axial β-chloroethyl group with the synaxial hydrogens apparently explains the larger conformation energy difference of N-mustard diastereomers over diethylamino epimers group as reported by Bentrude and coworkers.[2] Similarly, in 2-oxo-[bis-(2-chloroethylamino)]-4,6-dimethyl-1,3,2-oxazaphosphorinane, the exocyclic nitrogen with a sum of bond

Table VI
^{13}C Chemical Shifts[a]
(^{13}C-^{31}P coupling constants)[b]



Compound

carbon	5	6	7	8
6	24.04	23.87	24.19	24.29
1	24.54	24.17	24.76	24.75
2	25.50	26.43	28.07	28.07
5	32.84	32.91	33.30	33.35
	(8.24)	(6.03)	(9.65)	(7.64)
3	41.40	40.01	40.66	41.51
	(5.63)	(11.86)	(2.0)	(9.65)
7	71.29	72.26	47.94	47.19
	(5.63)	(6.03)	(3.0)	(0)
4	80.90	82.56	80.57	83.0
	(4.32)	(7.24)	(5.83)	(6.43)

a. Chemical shifts in parts per million from TMS.
b. In Hertz.

Table VII. IR P=O Stretching Frequencies (cm^{-1} of 5-8

Compd	Medium		
	KBr	CCl_4	$CDCl_3$
5	1266	1250	1240
6	1254	1245	1240
7	1228	1240	1215
8	1245	1250	1250.1210

angles of 359.8° fails to adopt an electronically optimal geometry due to the severe syn-1,3 diaxial interaction.[25c]

Configuration and Conformation of 5-8 in Solution

Compound 5,5-dimethyl-2-(dimethylamino)-2-oxo-1,3,2- oxazaphosphorinane was reported to exist in a chair conformation with predominately (65%) axial dimethylamino group, while the dioxa analogue[19a] exists with predominately (85%) equatorial dimethylamino group. As explained above, the bis(2-chloroethylamino) mustard group is sterically even bulkier than the dimethylamino group and the latter is expected to exist predominantly in the equatorial position of both dioxaphosphorinane and oxazaphosphorinane rings.

Thus in the dioxa system *5* and *6*, this steric effect appears to largely determine the conformation. Phosphorinane *5* is almost entirely in a chair form with an equatorial mustard group, and *6* is in a twist boat form *6a* with a pseudoequatorial group. It is presumably the steric interaction of the axial N-mustard group with the syn-axial H-1 and H-4 protons in the chair form that drives the conformation into the twist-boat conformation *6a* with the N-mustard group in the sterically favorable pseudoequatorial position. Similar steric interactions are responsible for the conformations of *7/8*. In the oxaza system *7* and *8*, however, the steric effect is less important presumably due to the longer endocyclic nitrogen bond relative to the endocyclic oxygen bonds in the dioxaphosphorines. Thus *7* favors a chair conformation with an equatorial mustard group, while *8* exists in both chair and twist-boat conformations.

Anticancer Activity

Low anticancer diastereoselectivity between diastereomeric pairs and low selectivity between oxaza and dioxaphosphorinanes *5-8* are observed. This may be a result of poor drug transfer due to the introduction of the hydrophobic moiety in addition to specificity of the mixed-function oxidase.[29,30] In addition, as pointed out by Bentrude et al.,[2] cyclophosphamide and its cyclic metabolites should be readily able to adopt the chair or twist conformations most advantageous to oxidation, ring opening of the 4-OH derivatives, or transport. Thus, due to this inherent flexibility of the heterocyclic six-membered ring, all diastereomers show essentially no difference in terms of diastereoselectivity.

It might be hypothesized that an analogous lipophilicity - activity relationship might exist in the cyclophosphamide series as was found for the nitrosourea using QSAR analysis.[31] The answer to this question can only be provided by

Table VIII. Anticancer Activity Against P-388 Leukemia in CD_2F_1 Male Mice.

COMPD.	DOSE(mg/kg)	% T/C[a]	% ILS[a]
5	25	137	37
	75	152	52
6	25	146	46
	75	156	56
7	25	152	52
	75	160	60
8	25	152	52
	75	167	67

[a] Antileukemic experiments were initiated on day 0 by IP implanation into the male CD_2F_1 mice of 10^6 P388 acites cells according to the NCI protocol(ref. 23). Drug treatment was commenced on day 1 and consisted of nine daily IP injections (days 1-9). Experiments were terminated when no mice remained alive. All-treated groups consisted of six mice and the leukemia group consisted of ten mice. The mice were observed daily, and the antileukemic activity of each compound was compared on the basis of the T/C criterion, where T represents the mean survival time of the treated group and C the mean survival time of the tumor-bearing control group. A value of T/C > 125 is usually considered to be the minimum requirement for a compound to be considered as active (ref. 23). The percent increase in life-span (ILS%) was calculated from the formula [(T-C)/C]x100. Clearly, the larger the value of the ILS, the more promising is the compound as an anticancer drug.

analogous QSAR analysis using a large number of cyclophosphamide derivatives possessing various lipophilicities.

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Supplementary Material Available Table of positional parameters, temperature factors for 7, $C_{11}H_{21}PO_2N_2Cl_2$ (6 pages). Ordering information is given on any current masthead page.

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