O-Imino Esters of N,N-Bis(2-chloroethyl)phosphorodiamidic Acid. Synthesis, X-ray Structure Determination, and Anticancer Evaluation

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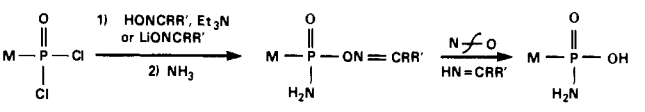
Nine representatives of the title series of compounds [$(ClCH_2CH_2)_2NP(O)(NH_2)ON{=}CRR'$] were synthesized as potential anticancer prodrugs, based on the possibility of enzymatic reduction of the N–O bond to release the known cytotoxic agent phosphoramide mustard [**1**, $(ClCH_2CH_2)_2NP(O)(NH_2)OH$]. The dimethyl derivative (**2**, R = R′ = CH_3) exhibited a statistically significant, albeit low, level of anti-L1210 activity in mice. Derivative **2**, which was shown by ^{31}P NMR measurements to be very stable toward hydrolysis at 37 °C over a pH range of 5.7–7.4 ($\tau_{1/2} \simeq$ 7–8 weeks), gave colorimetrically detectable amounts of alkylating material upon incubation with mouse liver slices: ~3–5% conversion after 20 min at 37 °C. A single-crystal X-ray study of **2** revealed an unusual hydrogen-bonded "ladder" and a very similar steric relationship for the NCH_2CH_2Cl and $ON{=}CCH_3$ moieties.

The anticancer activity of the prodrug cyclophosphamide results from its liver microsomal oxidation and the subsequent release of phosphoramide mustard [**1**, $(ClCH_2CH_2)_2NP(O)(NH_2)OH$], which is a lethal bis(alkylating) agent.[1-3] In view of the fact that these and other N-phosphorylated derivatives of *N,N*-bis(2-chloroethyl)amine constitute a relatively successful class of oncolytic compounds,[2,3] we have investigated new types of cyclophosphamide analogues,[3] as well as hydrolytically labile *O*-aryl[4] and *O*-triorganosilyl[5] esters of **1** as alternative precursors of **1** in vivo; however, none of the congeneric acyclic esters[4,5] gave exceptionally promising results. The present report concerns a brief study of *O*-imino esters of **1**, which feature a structurally novel P(O)ONC moiety that could be the locus of a reductive mechanism for the in vivo release of **1** (cf. Scheme I). Previous results obtained with "*N*-hydroxycyclophosphamide"[6] have indicated that microsomal enzymes are capable of mediating the reduction of an N–O bond; this mode of generation of **1** from its *O*-imino esters would exemplify a new approach to bioreductive alkylating agents.[7]

Chemistry and X-ray Results. The reaction of $Cl_2P(O)M$ [M = $N(CH_2CH_2Cl)_2$] with 1 equiv of various dialkyl oximes (Scheme I) was followed by ammoniolysis to give 30–80% isolated yields of the *O*-imino esters **2–8**. Aryl-substituted products **9** and **10** were prepared similarly but in very low yield (9 and 0.4%, respectively). Numerous attempts to isolate the hydrogen-substituted compounds **11** and **12** were unsuccessful[8a] and led instead to formation of the corresponding nitrile (R′CN) by a precedented[9] 1,2-elimination. It is also noteworthy that replacement of the oxime starting material with either HONHR, $HONR_2$, or HONHC(O)R repeatedly failed to provide evidence for the formation of the corresponding saturated analogues of **2–10**, even though the C=N moiety in **2** and **3** could be quantitatively reduced with $NaCNBH_3$ to give a HC–NH linkage.[8b]

Dimethyl derivative **2**, which was found to exhibit anticancer activity (vide infra), was selected for a single-crystal X-ray structure determination in order to examine the novel P(O)ON=C substructural unit and to provide additional details regarding the geometry of phosphoramidic mustards, which has been a subject of considerable interest.[10,11] Single crystals of racemic **2** ($C_7H_{16}N_3O_2PCl_2$) suitable for X-ray analysis were prepared by recrystallization from a mixture of chloroform and petroleum ether. Data were collected on a crystal of dimensions 0.12 × 0.12 × 0.28 mm using an automated four-circle diffractometer. The structure was solved by a combination of phasing techniques. The positions of the phosphorus and chlorine atoms were determined by direct methods, while successive least-squares refinements and Fourier syntheses revealed the positions of the remaining non-hydrogen atoms. A preliminary anisotropic least-squares refinement of all

Scheme I



	R	R′
2	Me	Me
3	Me	Et
4	Et	Et
5	i-Pr	i-Pr
6	i-Bu	i-Bu
7	$(CH_2)_4$	
8	$(CH_2)_5$	
9	Me	Ph
10	Ph	Ph
11	H	Me
12	H	Ph

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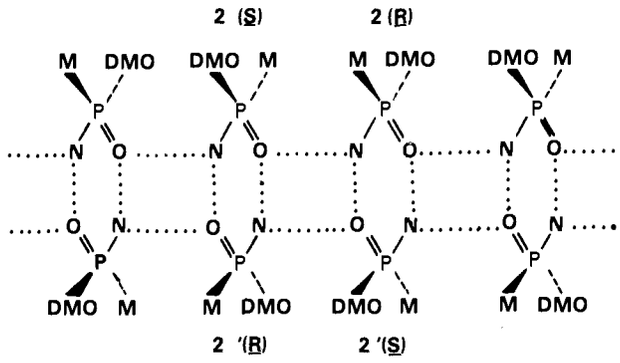

Figure 1. Schematic representation of the hydrogen bonds (~ 2.94 Å) in the crystal structure for racemic **2** (cf. text): M = $N(CH_2CH_2Cl)_2$, DMO = $ONC(CH_3)_2$, N = NH_2; *R* and *S* refer to the absolute configuration at phosphorus.

atoms, except hydrogen, gave a conventional agreement factor of $R = 0.077$ for 2562 observed reflections with $I \geq 3\sigma(I)$. Tables of bond distances, angles, and dihedral angles are available as supplementary material (see paragraph at end of paper concerning supplementary material).

Each molecule participated in four hydrogen bonds that were almost identical in length (N···O distance $\simeq$ 2.94 Å). As shown schematically in Figure 1, the NH_2 group served as the donor in two hydrogen bonds, while each phosphoryl oxygen atom was the acceptor in *two* hydrogen bonds[12] (^1H, ^{13}C, and ^{31}P NMR spectra failed to indicate the existence of persistent hydrogen bonding in solution, on NMR time scale). Molecule **2** was hydrogen bonded to a centrosymmetrically related counterpart to form dimers **2**···**2'**. In addition, each molecule was hydrogen bonded to two adjacent molecules having the opposite absolute configuration at phosphorus, forming a "ladder" of hydrogen bonds. The solid-state structure was also interesting with regard to the orientations of the $N(CH_2CH_2Cl)_2$ and $ON{=}C(CH_3)_2$ substituents in **2**. Parallel to the ladder of hydrogen bonds, there were two rows of chlorine atoms and four rows of *alternating* chlorine atoms and methyl carbon atoms. The distances from the phosphorus atom to the two chlorine atoms and to one methyl carbon atom were approximately equal (~5.0 Å). It thus appeared that an $ON{=}CCH_3$ moiety had "substituted" for an NCH_2CH_2Cl moiety in the crystal structure. Final refinement of the model including hydrogen atoms will be carried out, and a structural paper will be published elsewhere.

The nonemzymatic hydrolysis of the *O*-imido esters of **1** was gauged by ^{31}P NMR kinetic analyses[13,14] of **2**. Buffered samples of **2** at pH 7.4 and 5.7 gave $\tau_{1/2} \simeq$ 7–8 weeks at 37 °C; hence, cleavage of the P–O bond to give **1**, as well as cleavage of the P–N bond to give $(ClCH_2CH_2)_2NH$, were very slow "activation" processes, relative to normal pharmacokinetic time scales. ^{31}P NMR measurements also indicated that **2** did not react with protein (bovine γ-globulin) during 30 h of contact at pH 7.4, 25 °C. The marked resistance of **2** toward nucleophilic displacement of $HON{=}C(CH_3)_2$ was in opposition to the well-known lability of structurally analogous *N*-hydroxysuccinimide esters of carboxylic acids. It was assumed that the chemical inertness found for **2** was typical of the other *O*-imino esters of **1**.

Biological Results

Compound **2** was separately incubated (37 °C) in phosphate-buffered saline with freshly prepared slices (~60 μmol of **2**/g of liver) that were obtained from male and female mice. Parallel incubations were carried out with the cyclohexylammonium salt of phosphoramide mustard (**1**·CHA), in the presence of liver slices, and with **2** in the absence of liver slices. Aliquots were removed periodically, and the alkylating activity due to **1** [*or* $HN(CH_2CH_2Cl)_2$ *or both of these fragments*] was quantified by a procedure that was a modification of two previously reported[15,16] colorimetric methods, which employed 4-(*p*-nitrobenzyl)pyridine (NBP). While the NBP-derived absorbance values measured at 575 nm demonstrably provided a relatively sensitive and color-stable indicator of the concentration of **1**, the extent of the liver-mediated conversion of **2** into **1** was quite low, which precluded detailed time-course studies. On a *qualitative* basis, it was nevertheless apparent that (1) increasing concentrations of **1** were obtained by the incubation of **2** with liver slices for 5, 10, and 20 min, (2) male mouse livers afforded ~ 4–5% conversion after 20 min, while female mouse livers were, at most, approximately one-half as effective, and (3) compound **2** was *not* subject to adventitious formation of **1** by hydrolysis during either the incubation or NBP assay. The latter point was also established for compounds **3** and **6**. Incubation of **3** with female mouse liver for 20 min led to only ~0.5–1% conversion, which was the lowest level of reaction that could be detected. A similar result (~1% conversion) was obtained for the oxidative "activation" of cyclophosphamide.

Anticancer activity was evaluated according to standard screening protocols maintained at Otsuka Pharmaceutical Co. for L1210 lymphoid leukemia. Groups of seven male BDF_1 mice were inoculated intraperitoneally with 1×10^5 L1210 cells and were then given single intraperitoneal injections of selected test compounds in corn oil at doses of 3, 10, and 30 mg/kg. At the latter dosage, compounds **2**–**4** led to statistically significant, albeit low, activity (29, 11, and 13% increased life span, respectively), whereas compounds **6** and **7** were inactive in this test system. Independent screening of **2** by the National Cancer Institute, according to standard protocols,[18] confirmed its moderate anti-L1210 activity (maximum test/control value = 139% at 62 mg/kg) and further revealed its general toxicity (lethality) at higher doses (≥125 mg/kg). On the other hand, these NCI tests with **9** showed that it was inactive and virtually nontoxic over a wide dose range (31–750 mg/kg). For comparative purposes, we note that a single-dose injection (200 mg/kg) of **1**·CHA has given a 265% test/control value in the mouse L1210 evaluation system.[5]

In summary, the in vitro liver-mediated release of **1** from its *O*-imino esters and the in vivo anticancer activity of these esters were tentatively ascribed to the enzymatic reduction of the N–O bond; however, hydrolytic "activation" (production of **1**) via a phosphoesterase cannot be ruled out on the basis of the present data. The low levels or absence of anti-L1210 activity found for the *O*-

(12) For comparative purposes, we note that the crystal structure of an antamanide analogue [*cyclo*-(Gly-L-Pro-L-Pro-Gly-L-Pro-L-Pro)] also possesses a coherent system of hydrogen bonding that features an intermolecular bifurcated hydrogen bond; however, in this case it is maintained by an amide hydrogen that is shared with two carbonyl oxygens [Czugler, M.; Sasvari, K.; Hollosi, M. *J. Am. Chem. Soc.* **1982**, *104*, 4465].

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imino esters of 1 and our inability to synthesize analogous esters with sp^3 nitrogen bonded to oxygen suggested that other prodrug strategies for release of 1 may be more fruitful.

Experimental Section[19]

Synthesis of *O*-Imino Esters of Phosphoramide Mustard. Method A. A solution of the oxime (10 mmol) in CH_2Cl_2 (20 mL) was added dropwise to a solution of bis(2-chloroethyl)-phosphoramidic dichloride (10 mmol) and Et_3N (10 mmol) in CH_2Cl_2 (20 mL), and the mixture was stirred for 3–72 h at room temperature. Ammonia was bubbled through the reaction mixture for 1 h at 5–10 °C, and the solution obtained by suction filtration was concentrated on a rotary evaporator. The resultant residue was chromatographed on silica gel using $CHCl_3$–MeOH (9:1) as the eluent, unless specified otherwise, and the product was further purified by crystallization, if possible. Products that gave unacceptable combustion analyses were judged to be ≥95% pure based on ^1H NMR spectra [60 MHz, $CDCl_3$ with $Si(CH_3)_4$)].

Method B. A cold (ca. −20 °C) solution of the oxime (10 mmol) in THF (10 mL) was treated with a hexane solution of *n*-BuLi (10 mmol), and the mixture was stirred for 2 h before it was added by syringe to a cold (ca. −20 °C) solution of bis(2-chloroethyl)-phosphoramidic dichloride (10 mmol) in THF (10 mL). The temperature of the reaction mixture was adjusted to 5–10 °C after 3 h, and the mixture was then treated with NH_3 and further processed as in method A. Pertinent details are listed below.

2-Propanone *O*-[amino[bis(2-chloroethyl)amino]phosphinyl]oxime (2): method A; R_f 0.52; yield 80%; mp 86–88 °C ($CHCl_3$–petroleum ether); ^1H NMR δ 3.9–3.07 (m, 10), 1.98 (s, 6, 2 CH_3); ^{13}C NMR (25 MHz) δ 163.85 (d, J_{CP} = 12.82 Hz, C=N), 49.53 (d, J_{CP} = 4.3 Hz, 2 NCH_2), 42.37 (s, 2 CH_2Cl), 21.64 (s, 1 CH_3), 16.50 (s, 1 CH_3). Anal. ($C_7H_{16}N_3O_2PCl_2$) C, H, N.

2-Butanone *O*-[amino[bis(2-chloroethyl)amino]phosphinyl]oxime (3): method A; R_f 0.59; yield 80%; oil; ^1H NMR δ 3.87–3.0 (m, 10), 2.32 (q, 2, CH_2CH_3), 1.95 (s, 3, CH_3), 1.13 (t, 3, CH_2CH_3). Anal. ($C_8H_{18}N_3O_2PCl_2$) C, H, N.

3-Pentanone *O*-[amino[bis(2-chloroethyl)amino]phosphinyl]oxime (4): method A; R_f 0.56; yield 75%; oil; ^1H NMR δ 3.87–3.07 (m, 10), 2.48 (q, 2, 1 CH_2CH_3), 2.40 (q, 2, 1 CH_2CH_3), 1.15 (t, 3, 1 CH_3), 1.13 (t, 3, 1 CH_3).

2,4-Dimethyl-3-pentanone *O*-[amino[bis(2-chloroethyl)-amino]phosphinyl]oxime (5): method B; R_f 0.21 (CH_2Cl_2–MeOH, 96:4); yield 40%; yellow oil; ^1H NMR δ 3.83–2.97 (m, 10), 2.97–2.37 (m, 2, 2 CH), 1.17 (d, 12, 4 CH3). Anal. Calcd for $C_{11}H_{24}N_3O_2PCl_2$: C, 39.77; H, 7.28; N, 12.65. Found: C, 41.65; H, 7.91; N, 10.55.

2,6-Dimethyl-4-heptanone *O*-[amino[bis(2-chloroethyl)-amino]phosphinyl]oxime (6): method A; R_f 0.48 ($CHCl_3$–MeOH, 96:4) yield 65%; off-white solid; mp 49 °C (crystallized under vacuum); ^1H NMR δ 3.82–2.92 (m, 10), 2.42–1.72 (m, 6, 2 CH_2CH), 1.05–0.78 (m, 12, 4 CH_3). Anal. Calcd for $C_{13}H_{28}N_3O_2PCl_2$: C, 43.33; H, 7.85; N, 11.66. Found: C, 45.31; H, 8.50; N, 11.12.

Cyclopentanone *O*-[amino[bis(2-chloroethyl)amino]-phosphinyl]oxime (7): method A; R_f 0.59; yield 62%; oil; ^1H NMR δ 3.87–3.07 (m, 10), 2.77–2.20 (m, 4, 2 CH_2C=N), 1.97–1.60 (m, 4, 2 CH_2CH_2C=N). Anal. ($C_9H_{18}N_3O_2PCl_2 \cdot {}^1/_2\ H_2O$) C, H; N: calcd, 13.51; found, 12.95.

Cyclohexanone *O*-[amino[bis(2-chloroethyl)amino]-phosphinyl]oxime (8): method B: R_f 0.27 (CH_2Cl_2–MeOH, 96:4); yield 30%, oil; ^1H NMR δ 4.13–3.07 (m, 10), 2.73–2.10 (m, 4, 2 CH_2C=N), 1.90–1.47 (br s, 6, 3 aliphatic ring CH_2).

(19) Routine synthetic procedures and NMR spectroscopic details have been reported.[6]

Methyl phenyl ketone *O*-[amino[bis(2-chloroethyl)-amino]phosphinyl]oxime (9): method A; R_f 0.36; yield 9%; yellow oil; ^1H NMR δ 7.83–7.07 (m, 5, aromatic), 3.87–3.03 (m, 10), 2.37 (s, 3, CH_3). Anal. ($C_{12}H_{18}N_3O_2PCl_2$) H; C: calcd, 42.61; found, 44.12; N: calcd, 12.43; found, 11.82.

Diphenyl Ketone *O*-[amino[bis(2-chloroethyl)amino]-phosphinyl]oxime (10): method A; R_f 0.3–0.4 ($CHCl_3$–MeOH, 99:1) yield 0.4%; oil; ^1H NMR δ 7.62–6.85 (m, 10, aromatic), 3.85–3.25 (m, 10).

Incubations with Liver Slices and Assay for Compound 1. Male and female Balb/C mice (~25 g) were sacrificed by cervical dislocation, and the livers were immediately removed for manual slicing and temporary storage of the slices in cold saline. Saline-washed slices that corresponded to the entire liver of either a male or female mouse were added to 25-mL Erlenmeyer flasks that contained thermally equilibrated (37 °C, shaker-incubator) phosphate-buffered saline (PBS, pH 7.4) solutions (3 mL) of either the test compound (14 mM) or the cyclohexylammonium salt of phosphoramide mustard (1·CHA, 1 mM). The solutions of 1·CHA were prepared immediately prior to use to minimize hydrolytic loss of alkylating activity.[13,14] Suspensions of the liver slices in PBS were similarly prepared and then incubated to provide solution samples for zeroing the spectrophotometer; parallel incubations of PBS solutions of **2**, **3**, or **6** (14 mM) without liver slices were used to rule out the adventitious hydrolytic formation of 1. Aliquots (0.1 mL) for assay of alkylating activity were centrifuged, and the supernatant was then reacted with a solution (0.5 mL) of 5%, w/w, NBP in acetone for 2 h at 37 °C. A solution (0.8 mL) of 50%, v/v, Et_3N in acetone was added to produce a blue color that was quantified by measuring the absorbance (*A*) value at 575 nm. This NBP assay allowed for the detection of ≥50 nmol of 1 in the aliquots removed from the incubation mixtures. The percentage of "conversion" was defined as $([\mathbf{1}\cdot CHA]_{initial}/[\text{test compd}]_{initial})\ (A_{\text{test compd}}/A_{1\cdot CHA}) \times 100$.

NMR Studies. NMR samples of **2** (15 mg/2 mL) at pH 7.4 (1 M 2,6-dimethylpyridine) and 5.7 (1 M Bistris[14]) were kept at 37 °C, and ^{31}P NMR spectra were recorded periodically over ~3 weeks. Pseudo-first-order rate constants were measured by comparison of the relative intensities of the signal for **2** and the upfield signals due to inorganic phosphorus products.[14]

Compound **2** (5.6 mg, 20 μmol) was sonicated into a mixture of imidazole buffer (0.7 mL of 1 M, pH 7.4) and aqueous NaCl (1.0 mL of 0.2 M). The ^{31}P NMR spectrum was recorded, bovine γ-globulin (30 mg) was added, and another spectrum was recorded. No spectral changes were apparent after 30 h at 25 °C. The sample was passed through a Sephadex G-100 column, and the recovered protein, after lyophilization, had no detectable phosphorus-containing material (<0.5 μmol of phosphorus).

Acknowledgment. This investigation was supported in part by Research Grants CA-21345 (G.Z. and S.M.L.) and DE-05030 (S.T.) from the National Institutes of Health, PHS/HHS. The X-ray structure determination described in this paper was carried out at the National Bureau of Standards.

Registry No. 1·CHA, 87040-44-6; **2**, 87040-45-7; **3**, 87040-46-8; **4**, 87050-13-3; **5**, 87040-47-9; **6**, 87040-48-0; **7**, 87040-49-1; **8**, 87040-50-4; **9**, 87040-51-5; **10**, 87040-52-6; $Cl_2P(O)N(CH_2CH_2Cl)_2$, 127-88-8; $HONCMe_2$, 127-06-0; HONCMeEt, 96-29-7; $HONCEt_2$, 1188-11-0; $HONC(i\text{-}Bu)_2$, 52435-41-3; $HONC(CH_2)_4$, 1192-28-5; HONCMePh, 613-91-2; $LiONC(i\text{-}Pr)_2$, 87040-53-7; $LiONC(CH_2)_5$, 87040-54-8.

Supplementary Material Available: A positional labeling scheme and two tables of bond distances, angles, and dihedral angles for **2** (4 pages). Ordering information is given on any current masthead page.