in the range of 9–11 days for all experiments.

Cholinesterase Assay. Acetylcholinesterase activity in whole blood was determined as previously described.[16] Whole blood (20 μL) was collected by retroorbital puncture from $B_6D_2F_1$ mice and diluted into 10 mL of phosphate buffer (2 mM, pH 7.4, 37 °C) containing bromthymol blue (100 mg/L). The solution was divided into two portions; acetylcholine chloride (0.1 mL, 0.83 M) was added to one portion, and water (0.1 mL) was added to the other portion. An aliquot of each solution was transferred to a cuvette; the sample cuvette contained acetylcholine and the other was used as the reference cuvette in a thermostated spectrophotometer cell (37 °C). Net absorbance (620 nm) was measured at 1-min intervals for 20 min; absorbance change was linear over this interval, and enzymatic activity was determined from the rate of change in absorbance obtained from linear regression. Baseline activity was measured in each of three mice; drugs were suspended in 2% (carboxymethyl)cellulose and injected ip 1 h later. Inhibition of enzyme activity was determined from the ratio of enzyme activity following drug administration and the baseline level, with each animal serving as its own control.

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Synthesis, Activation, and Cytotoxicity of Aldophosphamide Analogues

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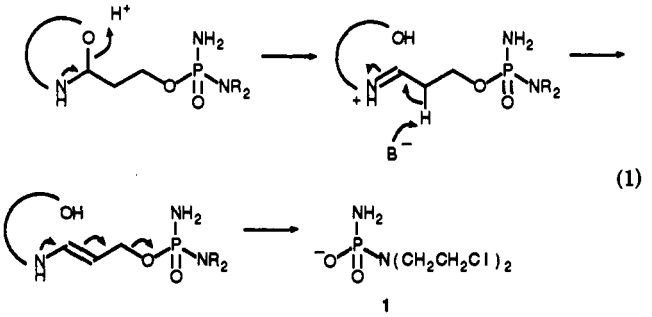
A series of perhydrooxazine analogues of aldophosphamide has been prepared, and their ^{31}P NMR kinetics and in vitro cytotoxicity have been evaluated. These compounds were developed on the basis of the idea that ring opening and tautomerization to an enamine intermediate might provide a mechanistic alternative to the β-elimination reaction for release of phosphoramide mustard. The 4,4,6-trimethyltetrahydro-1,3-oxazine moiety was selected on the basis of its rapid rate of iminium ion generation and relatively slow rate of hydrolysis. These analogues underwent phosphorodiamidate release by three distinct mechanisms: hydrolysis to aldophosphamide and subsequent β-elimination; cyclization to produce the 4-hydroxycyclophosphamides, which release phosphorodiamidate by ring opening and elimination; and tautomerization to the enamine with rapid expulsion of phosphorodiamidate. Kinetic studies demonstrated that hydrolysis to the aldehyde contributed minimally to the overall activation process and that the enamine pathway represented the major route of activation. For those analogues that could undergo cyclization, this pathway competed effectively with enamine release, and these analogues were essentially equivalent to their 4-hydroxycyclophosphamide counterparts in cytotoxicity. A series of tetra-N-substituted phosphorodiamidates that cannot undergo cyclization was prepared to explore the effects of cyclization on the cytotoxicity of these analogues. The tetrakis(chloroethyl)phosphorodiamidates were highly potent in vitro against both cyclophosphamide-sensitive and -resistant L1210 and P388 cell lines, and one of these analogues had significant antitumor activity against L1210 leukemia in vivo. These results demonstrate that the enamine mechanism provides a viable pathway for delivery of phosphorodiamidates and that this approach can be used to deliver phosphorodiamidates that are non-cross-resistant in cyclophosphamide-resistant cell lines.

Introduction

Cyclophosphamide and its analogues are important clinical agents in the treatment of cancer.[1] The development of drug resistance and the incidence of host toxicities prompted the development of a synthetic program directed at analogues designed to minimize these effects. We have reported the synthesis and evaluation of activated substituted cyclophosphamides that release phosphoramide mustard (PDA, 1) via β-elimination from structurally modified aldehydes.[2] Another approach, however, involves the synthesis of aldehyde analogues that may undergo activation by a different mechanism. In particular, we were attracted by the possibility that elimination of PDA could be driven by an enamine intermediate, generated from an appropriately substituted nitrogen derivative of the aldehyde (eq 1). Mechanistic precedent for this strategy exists in the well-established specific amine catalysis of carbonyl β-elimination reactions.[3] We



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report here the synthesis, activation kinetics, and antitumor activity of several aldophosphamide analogues and confirm that the enamine pathway provides a viable alternative for PDA release.

Results and Discussion

Aldehyde Derivatives. Efforts were directed initially to the identification of aldehyde analogues that would have

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(2) Borch, R. F.; Canute, G. W. Synthesis and antitumor properties of activated cyclophosphamide analogues. *J. Med. Chem.*, preceding paper in this issue.

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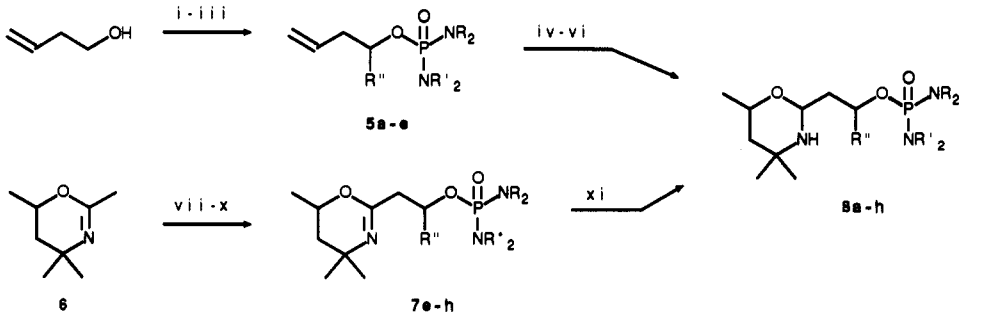
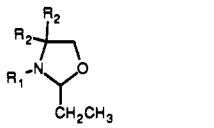
Scheme I[a]



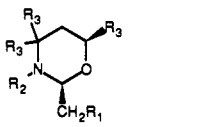
	R	R'	R"		R	R'	R"
a:	CH_2CH_2Cl	H	H	e:	CH_2CH_2Cl	H	Ph
b:	CH_2CH_3	CH_2CH_3	H	f:	CH_2CH_3	H	Ph
c:	CH_2CH_2Cl	$(CH_2CH_2)_2O$	H	g:	CH_2CH_2Cl	$(CH_2CH_2)_2O$	Ph
d:	CH_2CH_2Cl	CH_2CH_2Cl	H	h:	CH_2CH_2Cl	CH_2CH_2Cl	Ph

[a] (i) BuLi, THF; (ii) $R_2NP(O)Cl_2$; (iii) R'_2NH; (iv) O_3, CH_2Cl_2; (v) Me_2S; (vi) 4-methyl-4-amino-2-pentanol; (vii) BuLi, THF; (viii) R"CHO; (ix) $R_2NP(O)Cl_2$; (x) R'_2NH; (xi) $NaBH_4$, pH 6–8.

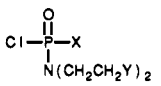
Chart I



2a: R_1=R_2=H
2b: R_1=H, R_2=CH_3
2c: R_1=CH_2CH_2OH, R_2=H
2d: R_1=$COCH_3$, R_2=H



3a: R_1=R_2=H, R_3=CH_3
3b: R_1=CH_3, R_2=$COCH_3$, R_3=H
3c: R_1=R_2=H, R_3=CH_3
3d: R_1=R_3=CH_3, R_2=H
3e: R_1=CH_2Ph, R_2=H, R_3=CH_3



4a: X=Y=Cl
4b: X=$N(CH_2CH_2Cl)_2$, Y=Cl
4c: X=Cl, Y=H

sufficient stability in aqueous solution that enamine formation could compete with hydrolysis. Oxazolidines **2a–d** and perhydrooxazines **3a–e** (Chart I) were prepared by reaction of the respective aldehydes with the corresponding amino alcohols; acetylation of **2a** and **3a** afforded amides **2d** and **3b**. The hydrolytic stability of these compounds in aqueous buffer (0.1 M phosphate in D_2O, pD 7.4, 20 °C) was assessed by ^1H NMR. Hydrolysis of compounds **2a–c** and **3a** was essentially complete within 5 min; in contrast, hydrolysis of amides **2d** and **3b** was negligible after 2 h. Methyl substitution on the perhydrooxazine ring retarded the hydrolysis rates of **3c–e** compared to that of **3a**; reaction half-lives were ca. 4 h, and under these conditions (ca. 20 mM oxazine) the reaction proceeded to an equilibrium mixture of 1:4 oxazine:aldehyde. When hydroxylamine was added to **3a**, **3c**, or the equilibrium mixture from **3c** in buffer, conversion of aldehyde and oxazine to the oxime was complete within minutes. These results are consistent with a hydrolysis mechanism involving rapid and reversible ring opening of the perhydrooxazine with rate-limiting attack of water or hydroxide ion on the resulting iminium ion. The methyl groups presumably accelerate the ring-closure reaction and thus shift the initial equilibrium to the left, slowing the hydrolysis by decreasing the iminium ion concentration.

The dependence of hydrolysis rate on pH and buffer concentration was determined for **3d** and **3e** by using a spectrophotometric assay; the results are presented in Table I. Hydrolysis proceeds with a half-life of 80 min at pH 7.4, 37 °C, and the rate appears to be independent of buffer concentration. The pH–rate profile over the limited range examined is consistent with the mechanism proposed above. At pH >7.4 the hydroxide and iminium ion concentrations vary inversely with pH, so there is no net change in rate; in the pH range 6.8–7.4, the iminium ion concentration remains constant and the rate decreases with decreasing hydroxide ion concentration. These observations are similar to those reported for oxazolidine hydrolysis.[4] Given this optimal combination of rapid iminium ion generation and moderate hydrolysis rates for **3c–e**, a series of aldophosphamide derivatives **8** was then prepared.

Table I. Kinetics of Perhydrooxazine Hydrolysis (HEPES Buffer, 37 °C)

compd	pH	[HEPES], mM	$k_{hydr} \times 10^3$, min^{-1}
3d	7.4	12.5	8.8 ± 0.5
3d	7.4	25	8.5 ± 0.5
3d	7.4	50	8.6 ± 0.5
3e	6.8	25	4.2 ± 1.0
3e	7.0	25	6.5 ± 1.0
3e	7.4	12.5	8.6 ± 0.6
3e	7.4	25	9.4 ± 0.6
3e	7.4	50	9.5 ± 0.5
3e	7.8	25	8.2 ± 1.0

Synthesis. The approaches used for the synthesis of **8a–h** are outlined in Scheme I. Compounds **5a–d** were prepared by sequential reaction of 3-buten-1-ol with butyllithium, the appropriate phosphoramidic dichloride, and ammonia or the corresponding secondary amine. The double bond was ozonized and reduced with dimethyl sulfide, and the resulting aldehyde trapped in situ with 4-amino-4-methyl-2-pentanol in the presence of anhydrous potassium carbonate to give products **8a–d**. Cyclization

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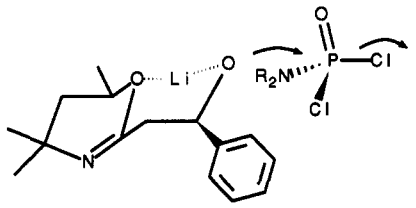

Figure 1. Stereoselective attack of coordinated lithium alkoxide on the phosphoramidic chloride.

of the intermediate aldehyde competed with oxazine formation for **8a** and, for the analogues where R″ ≠ H, cyclization was the exclusive reaction. Thus an alternative route was developed for the synthesis of analogues **8e–h**. Dihydrooxazine **6** was reacted with butyllithium,[5] and the resulting anion was treated sequentially with the appropriate aldehyde, the phosphoramidic dichloride, and ammonia or the requisite amine to give the substituted dihydrooxazines **7e–h**, which were reduced with sodium borohydride at pH 7 to give products **8e–h**.

The stereochemistry of these products warrants further comment. First it should be noted that although a number of stereoisomers may be produced in these reactions, all isomers of a given compound will ultimately release the identical cytotoxic species. Because the perhydrooxazine ring is generated under equilibrium conditions for both of these routes, the most stable ring conformation (C-2 and C-6 substituents equatorial) will be obtained. For those *unsubstituted* products in which phosphorus is chiral (**8a** and **8c**), a 1:1 mixture of diastereomers is obtained as expected. For those *substituted* products with chiral phosphorus (**8e–g**), the additional chiral center at the substituent carbon must be considered, and a 1:1:1:1 mixture of four diastereomers would be expected. However, the ^{31}P spectrum of the crude product **8e** showed the presence of four diastereomers in a ratio of 46:46:4:4. In order to determine where the stereoselectivity was arising, the intermediate resulting from condensation of the lithiooxazine with benzaldehyde (the intermediate for **8e**) was trapped with acetyl chloride. The ^{1}H spectrum of the crude product indicated the presence of two diastereomers in a 1:1 ratio, confirming that the stereoselectivity observed in **8e** must be arising in the phosphorylation reaction. Similarly, the lithium alkoxide of 1-phenyl-3-buten-1-ol was reacted with the phosphoramidic dichloride and ammonia to give the analogous phosphorodiamidate **5e** with no stereoselectivity, confirming that the presence of the perhydrooxazine moiety is essential for stereocontrol in this reaction.

The two major isomers of **8e** were separated and hydrolyzed/cyclized in aqueous buffer; the reactions were monitored by ^{31}P NMR. Each isomer initially generated the *same* 1:1 mixture of 4-hydroxy-6-phenylcyclophosphamides (−12.7 and −10.3 ppm), which subsequently equilibrated over 2 h to give a 3:1 ratio of 4-hydroxy isomers as previously shown for the 4-hydroxy-6-phenyl compounds.[2] These results confirm that the stereochemical relationship between the benzylic and phosphorus chiral centers is identical for the two major isomers in this reaction. A detailed analysis of 4-hydroxy-6-phenylcyclophosphamide stereochemistry is reported elsewhere;[2] on the basis of the ^{31}P chemical shifts of the **8e** hydrolysis products, it may be concluded that the configuration of the phosphorus center in **8e** will be identical with the

Table II. Kinetics of Ring Closure and PDA Release for Perhydrooxazines (HEPES Buffer, 37 °C)[a]

compd	[HEPES], M	rate constants × 10^3, min^{-1} k_{dis}[a]	k_1[b]	k_2[b]	k_3[b]
		pH 7.4			
8e	0.4	17.9	9.2	8.5	8.9
8e	0.3	16.0	7.1	8.7	7.4
8e	0.2	13.7	5.2	8.7	6.1
8a	0.4	33.2			
8c	0.4	32.0			
		pH 6.4			
8e	0.4	26.0	5.2	20.5	3.0
8e	0.3	20.8	2.9	18.0	2.1
8e	0.2	17.2	2.0	15.8	<1.0
8a	0.4	23.6			
8c	0.4	23.8			

[a] Rate constant for disappearance of **8** obtained from linear regression of ln (C_t/C_0) vs t, where C_0 and C_t are concentrations of **8** at times 0 and t, respectively. [b] See eq 2 for definition of rate constants.

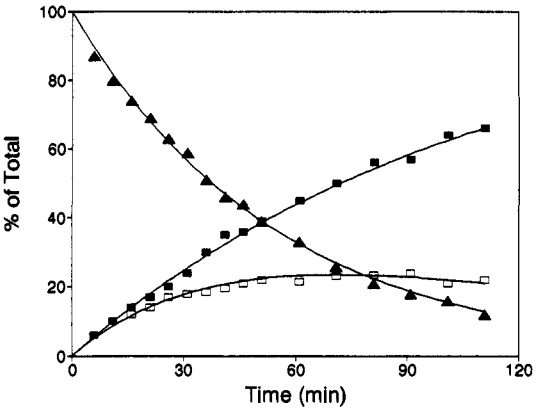

Figure 2. Activation of perhydrooxazine **8e** in HEPES buffer (0.4 M, pH 7.4, 37 °C). Data points were obtained from ^{31}P line intensities; the solid lines represent the best-fit values calculated by the Simplex algorithm according to eq 2: (▲) **8e**, (□) 4-hydroxy-6-phenylcyclophosphamide (**9**), (■) phosphoramide mustard (**1**) and its solvolysis products.

configuration of the benzylic carbon in the major products (e.g., *R,R* or *S,S*). It is proposed that conversion of the lithium alkoxide to **8e** occurs by successive displacements of chloride on phosphorus with inversion[6] and that the lithium alkoxide is oriented in a 6-membered ring with the phenyl group equatorial as a result of lithium stabilization by coordination with the oxazine oxygen (Figure 1). Approach of this alkoxide to the phosphoryl chloride occurs stereoselectively, with the bulky phosphoramide substituent oriented away from the phenyl group to minimize steric hindrance in the transition state.

Kinetics. The kinetics of PDA release were determined for the phenyl-substituted analogue by **8e** using ^{31}P NMR. The pathways available are summarized in Scheme II. Direct attack of hydroxide ion on the iminium ion (path a) leads to hydrolytic generation of aldophosphamide, which undergoes predominant cyclization to 4-hydroxy-6-phenylcyclophosphamide **9** and minor conversion to PDA via β-elimination. Intramolecular attack of the phosphoramide nitrogen on the iminium ion (path b) generates the cyclized 4-amino derivative, which would be converted to **9** via an elimination–addition reaction.[7,8]

(5) Meyers, A. I.; Nabeya, A.; Adickes, H. W.; Politzer, I. R.; Malone, G. R.; Kovalesky, A. C.; Nolen, R. L.; Portnoy, R. C. The synthesis of aldehydes from dihydro-1,3-oxazines. *J. Org. Chem.* **1973**, *38*, 36–56.

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(7) Kwon, C.-H.; Maddison, K.; LoCastro, L.; Borch, R. F. Accelerated decomposition of 4-hydroxycyclophosphamide by human serum albumin. *Cancer Res.* **1987**, *47*, 1505–1508.

Scheme II

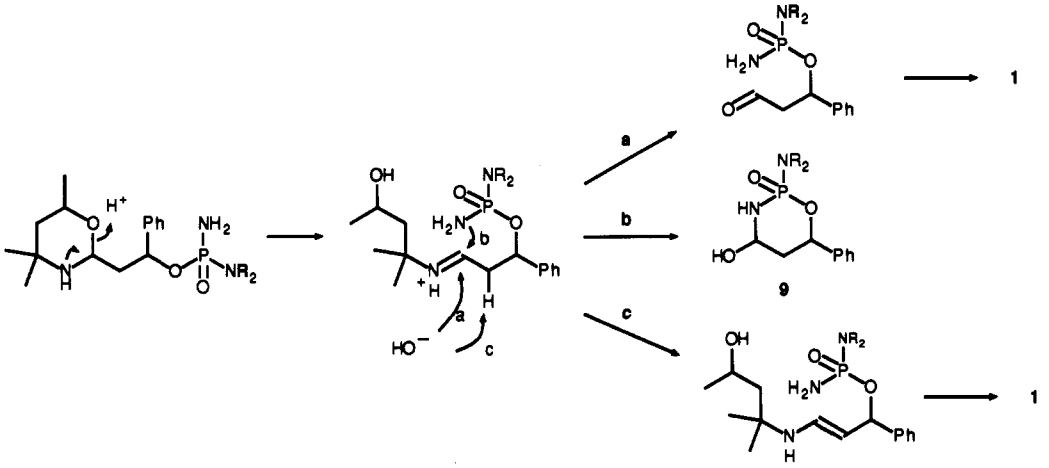

Table III. Antitumor Activity of Perhydrooxazinophosphorodiamidates in Vitro

no.	L1210/0,[a] μM	L1210/CP,[a] μM	RF[b]	P388/0,[a] μM	P388/CP,[a] μM	RF[b]
8a	21	145	6.9	19	92	4.8
8b	>200	>200		>200	>200	
8c	84	150	1.8	56	117	2.1
8d	7	19	2.7	5	10	2.0
8e	13	14	1.1	7	10	1.4
8f	>200	>200		>200	>200	
8g	28	38	1.4	16	22	1.4
8h	2	3	1.5	2	2	1.0
4-HC[c]	8	140	17	6	60	10

[a] LC_{99} values determined against wild-type L1210 or P388 cells, or cyclophosphamide-resistant L1210 or P388 cells, as described in the text. [b] Resistance factor = (LC_{99} resistant)/(LC_{99} wild type). [c] 4-Hydroperoxycyclophosphamide.

Finally, attack of hydroxide ion at the α-hydrogen (path c) results in tautomerization to the enamine, which can undergo direct expulsion of PDA. Resonance intensities of **8e**, **9**, and PDA + solvolysis products were measured at various times; the rate constants were determined by simultaneous nonlinear-least-squares fit of the kinetic data to the following equations by using the Simplex algorithm.[9]

$$\begin{array}{ccccc} \textbf{8e} & \xrightarrow{k_2} & \textbf{9} & \xrightarrow{k_3} & \textbf{1} \\ {\scriptstyle k_1}\downarrow & & & & \\ \textbf{1} & & & & \end{array} \qquad (2)$$

$$\text{fraction } \mathbf{8e} = e^{-(k_1+k_2)t}$$

$$\text{fraction } \mathbf{9} = \frac{k_2}{k_2 - (k_1 + k_2)}(e^{-(k_1+k_2)t} - e^{-k_3 t})$$

$$\text{fraction } \mathbf{1} = 1.0 - \mathbf{8e} - \mathbf{9}$$

The results of a representative experiment are shown in Figure 2, and the data are summarized in Table II.

It is apparent that conversion of oxazine to cyclized product is faster at lower pH; if this reaction were occurring via hydrolysis to the aldehyde, a *decrease* in rate with decreasing pH would be expected.[9] In a separate experiment, the aldehyde was generated directly by periodate cleavage of the diol resulting from hydroxylation of **5e**. This aldehyde underwent rapid cyclization and elimination, with cyclization representing 85% and 94% of product after 3 min at pH 7.4 and 6.4, respectively. If PDA were arising predominantly from hydrolysis to aldehyde and subsequent elimination, one would expect elimination:cyclization ratios (products **1** and **9**) of ca. 1:6 and 1:16 at pH 7.4 and 6.4, respectively. Examination of the data, however, indicates that these ratios are 1:1 and 1:4 at pH 7.4 and 6.4, respectively. These results suggest that path a does not contribute significantly to the generation of PDA. It is apparent from Figure 2 that at early time points, the rates of PDA generation and cyclization from **8e** are similar, suggestive of a direct pathway for PDA generation. Furthermore, when the direct reaction was excluded from the kinetic analysis (by assigning $k_1 = 0$; eq 2), a satisfactory fit of the data could not be obtained. The data in Table II confirms that the direct reaction is general-base-catalyzed, consistent with rate-limiting proton abstraction from the iminium ion followed by rapid expulsion of PDA as shown in path c.

The kinetic behavior of the unsubstituted perhydrooxazine **8a** was different from **8e** in several respects. First, there was no evidence of cyclization to 4-hydroxycyclophosphamide at pH 7.4 or 6.4; PDA and its hydrolysis products were the only products observed by ^{31}P NMR. Second, the rate of disappearance of **8a** was significantly faster than the rate of perhydrooxazine hydrolysis at pH 7.4 and 6.4 (Table I), confirming the existence of a nonhydrolytic route to PDA release. Finally, the disappearance of **8a** was slower at pH 6.4 than at 7.4, in contrast to the faster disappearance of **8e** observed at lower pH. The latter is presumably a consequence of faster cyclization at lower pH (Table II); because **8a** does not undergo appreciable cyclization, its disappearance is dominated by base-catalyzed tautomerization to the enamine. Identical rates of disappearance for **8a** and the tetra-N-substituted compound **8c**, which cannot cyclize, provides further evidence that disappearance of **8a** is independent of cyclization.

(8) Borch, R. F.; Getman, K. M. Base-catalyzed hydrolysis of 4-hydroperoxycyclophosphamide: Evidence for iminocyclophosphamide as an intermediate. *J. Med. Chem.* **1984**, *27*, 485.
(9) Borch, R. F.; Millard, J. A. The mechanism of activation of 4-hydroxycyclophosphamide. *J. Med. Chem.* **1987**, *30*, 427–431.

Table IV. Antitumor Activity of Perhydrooxazinophosphorodiamidates against L1210 Leukemia in Vivo

compd	% T/C (LTS)[a]		
	170 μmol/kg	340 μmol/kg	510 μmol/kg
8a	144	144	167
8c		113	113
8e	125	125	toxic
8g	100	100	111
8h	125	150	170
4-HC[b]	140	180	240 (3/10)

[a] Increased survival determined as described in the text; LTS = 30-day survivors. [b] 4-Hydroperoxycyclophosphamide.

In Vitro Cytotoxicity. The perhydrooxazine derivatives 8a–h were evaluated for cytotoxicity against wild-type (/0) and cyclophosphamide-resistant (/CP) L1210 and P388 cell lines in a clonogenic assay, using a 1-h drug exposure. The drug concentration that produced a 2-log reduction in clonogenic survival (LC_{99}) was determined from the linear portion of the log (surviving fraction) vs concentration curve; the results are reported in Table III. It is apparent that compounds lacking the bis(2-chloroethyl) group (8b and 8f) are devoid of antitumor activity. The oxazine derivative of aldophosphamide (8a) is somewhat less potent than 4-hydroperoxycyclophosphamide (4-HC) and offers no apparent advantage against CP-resistant cells. In contrast, phenyl analogue 8e is comparable to 4-HC against wild-type cells and is equipotent against both wild-type and resistant cells. However, the cytotoxicity of oxazine 8e is essentially equivalent to 4-hydroperoxy-6-phenylcyclophosphamide,[2] suggesting that the oxazine does not confer any apparent advantage over the cyclic compound.

The opportunity to substitute both phosphorodiamidate moieties represents a unique feature of these compounds in that cyclization will be blocked and more potent phosphorodiamidates may be available. Two analogue systems were evaluated: compounds with a single nitrogen mustard moiety (8c,g) and those with bis nitrogen mustard substitution (8d,h). When cyclization was blocked with a morpholine group, potency was reduced compared to that of the unsubstituted phosphorodiamidate analogue. In contrast, tetrakis(chloroethyl) analogues 8d and 8h showed excellent in vitro activity against both wild-type and resistant cell lines.

In Vivo Activity. Antitumor activity was assessed by using an intraperitoneal murine L1210 model and prolongation of survival as a measure of efficacy; the results are presented in Table IV. Analogues 8a and 8e were comparable in activity to the corresponding activated cyclophosphamides,[2] which may be a consequence of the facile cyclization reaction that they undergo. In contrast, those compounds containing a single mustard group that could not undergo cyclization (8c and 8g) were devoid of antitumor activity. Tetrakis analogue 8h showed significant antitumor activity at the higher doses tested. Comparison of this compound with tetrakis(2-chloroethyl)-phosphorodiamidic chloride,[10] which presumably generates the same alkylating species as 8h via hydrolysis of the P–Cl bond, reveals comparable activity at low dose (% T/C 128 at the MTD of 140 μmol/kg for the phosphorodiamidic chloride). However, the therapeutic efficacy of 8h is greater, because its reduced toxicity allows administration of higher drug doses. It is also of interest to note that *N,N*-diethyl-*N',N'*-bis(2-chloroethyl)phosphorodiamidic chloride is devoid of antitumor activity (% T/C 108 at the MTD of 670 μmol/kg),[10] consistent with our data for 8c and 8g, which release comparable (morpholino vs diethylamino) phosphorodiamidates.

Summary. We have shown that perhydrooxazine analogues of aldophosphamide can function as prodrugs for the release of phosphorodiamidates and that the enamine expulsion reaction is a major contributor to the release mechanism. For those compounds where the iminium ion can undergo cyclization, this pathway becomes the major competitive route for activation, and as a group these analogues offer little advantage over their 4-hydroxycyclophosphamide counterparts. Finally, a new class of tetrakis phosphoramide mustard prodrugs has been developed that are highly potent against both sensitive and cyclophosphamide-resistant leukemia cell lines and possess significant antitumor activity against L1210 leukemia in vivo.

Experimental Section

Melting points were determined on a Kofler block and are uncorrected. Elemental analyses were carried out by Galbraith Laboratories, Inc., Knoxville, TN. Ultraviolet spectral kinetics were carried out on a Beckman DU-8 instrument fitted with an automatic sample changer and thermostated cell chamber using the DU-8 Kinetics Module. NMR spectra were recorded on a Bruker WP-270 SY instrument. ^1H chemical shifts are reported in ppm downfield from Me_4Si (organic solvents) or $Me_3Si(CD_2)_2SO_3Na$ (D_2O); ^{31}P chemical shifts are reported in ppm from triphenylphosphine oxide in toluene-d_8 as coaxial reference. ^{31}P NMR kinetics were carried out as described previously.[7-9] Unless otherwise noted, all reactions were carried out under a nitrogen atmosphere, and organic extracts were dried over $MgSO_4$ and concentrated in vacuo.

Hydrolysis Kinetics. Hydrolysis of the model perhydrooxazines was followed spectrophotometrically by recording the increased absorbance of phenol red at 560 nm due to the pH change produced by the liberated amino alcohol. A stock solution containing 0.25 M HEPES buffer, phenol red (10 mg/100 mL), and KCl (μ = 0.20) was prepared. A similar stock solution containing water, phenol red, and KCl was also prepared. By mixing appropriate amounts of each stock solution, buffer concentration could be varied (total volume = 1.0 mL). All solutions were equilibrated at 37 °C. A solution of perhydrooxazine in DMSO (1:4 v/v) was added to the buffer solution, and an equal volume of DMSO was added to the reference cell; the molar ratio of perhydrooxazine:buffer was 0.1 in all experiments. Absorbance readings were taken over 1 h (<1 half-life) so that the reverse reaction of amino alcohol with aldehyde was insignificant. Rate constants were calculated from the slopes of linear plots of [ln $(A_\infty - A_t)$] against time, where A_t and A_∞ are the absorbance readings at times t and infinity, respectively. Infinity points could be obtained via (a) addition of an equimolar quantity of amino alcohol instead of perhydrooxazine or (b) quantitative release of the amino alcohol by treatment with hydroxylamine.

Synthesis of Oxazolidines 2 and Perhydrooxazines 3. The synthesis of 2-ethyl-4,4,6-trimethyltetrahydro-1,3-oxazine (3d) is representative. 4-Methyl-4-amino-2-pentanol (1.17 g, 0.01 mmol) was dissolved in CH_2Cl_2 (20 mL) containing anhydrous K_2CO_3 (2.76 g, 0.02 mol) and cooled to 0 °C, and a solution of propionaldehyde (0.90 mL, 0.0125 mol) in CH_2Cl_2 (4 mL) was added. Stirring was continued at room temperature for 2 h. The mixture was filtered and concentrated and the residue distilled to give 3d (1.32 g, 84%) as a colorless liquid: bp 45–46 °C (10 mm); ^1H NMR ($CDCl_3$) 4.16 (1 H, t), 3.74 (1 H, m), 1.55 (2 H, m), 1.43 (1 H, dd), 1.19 (3 H, d), 1.16 (3 H, s), 1.10 (3 H, s), 1.08 (1 H, dd), 0.97 ppm (3 H, t).

2-(2-Phenethyl)-4,4,6-trimethyltetrahydro-1,3-oxazine (3e) was prepared by reaction of hydrocinnamaldehyde with 4-methyl-4-amino-2-pentanol as described for 3d above. Distillation of the crude product afforded 3e (2.04 g, 87%) as a colorless liquid: bp 99–101 °C (0.75 mm); ^1H NMR ($CDCl_3$) 7.26 (5 H, m), 4.20 (1 H, t), 3.72 (1 H, m), 2.76 (2 H, m), 1.83 (2 H, m), 1.43 (1 H,

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dd), 1.17 (3 H, d), 1.13 (3 H, s), 1.10 (3 H, s), 1.08 ppm (1 H, dd).

N,N-Bis(2-chloroethyl)phosphoramidic dichloride (4a) was prepared as described[2] in 84% yield, mp 57–59 °C.

N,N,N',N'-Tetrakis(2-chloroethyl)phosphorodiamidic Chloride (4b). To a stirred suspension of 4a (5.18 g, 20.0 mmol) and bis(2-chloroethyl)amine hydrochloride (3.93 g, 22.0 mmol) in dry toluene (200 mL) was added triethylamine (6.13 mL, 44 mmol) at room temperature. The mixture was then refluxed for 16 h, cooled, and washed with 10% aqueous KH_2PO_4 (2 × 100 mL). The washings were extracted with ether (2 × 50 mL), and the combined extracts were concentrated to give a viscous oil. Purification by flash chromatography (hexanes/EtOAc 3:1) gave the product as a solid (4.01 g, 55%), which was recrystallized from ether/hexanes: mp 48–49 °C, ^{1}H NMR ($CDCl_3$) 3.71 (8 H, m), 3.56 ppm (8 H, m); ^{31}P NMR ($CDCl_3$) +0.66 ppm. Anal. ($C_8H_{16}Cl_5N_2OP$) C, H.

N,N-Diethylphosphoroamidic Dichloride (4c). Diethylamine (16.86 mL, 0.16 mol) was added dropwise with stirring to a cooled (0 °C) solution of phosphorus oxychloride (25.0 g, 0.16 mol) and triethylamine (25.0 mL, 0.18 mol) in CH_2Cl_2 (600 mL). This mixture was then allowed to warm to room temperature and stirred for 24 h. A 10% KH_2PO_4 solution in water (200 mL) was added at 0 °C. The mixture was warmed to room temperature and extracted with CH_2Cl_2 (3 × 50 mL). The organic extracts were washed with brine and concentrated; the resulting oil was distilled to give 4c (26.98 g, 87%) as a clear liquid: bp 75–76 °C (0.8 mm); ^{1}H NMR ($CDCl_3$) 3.32 (4 H, m), 1.23 ppm (6 H, t); ^{31}P NMR ($CHCl_3$) −7.91 ppm.

3-Butenyl N,N-Bis(2-chloroethyl)phosphorodiamidate (5a). *n*-Butyllithium (1.48 mL, 2.2 mmol) was added dropwise to 3-buten-1-ol (144 mg, 2.0 mmol) in THF (10 mL) at room temperature, and the solution was stirred for 30 min and then cooled to 0 °C. A solution of phosphoramidic chloride 4a (485 mg, 2.1 mmol) in THF (5 mL) was added rapidly, and stirring was continued for 30 min. Ammonia gas was then passed through the solution for 10 min and the resulting suspension warmed to room temperature and stirred for 2 h. The salts were removed by filtration through diatomaceous earth, and the filtrate was concentrated to give 5a as a yellow oil (510 mg, 93%) that could be used without further purification. An analytical sample was obtained by flash chromatography (CH_2Cl_2/acetone 1:1): ^{1}H NMR ($CDCl_3$) 5.79 (1 H, m), 5.15 (2 H, m), 4.04 (2 H, m), 3.64 (4 H, t), 3.43 (4 H, m), 2.78 (2 H, br s), 2.42 ppm (2 H, m); ^{31}P ($CHCl_3$) −9.30 ppm. Anal. ($C_8H_{17}Cl_2N_2O_2P$) C, H.

3-Butenyl N,N,N',N'-Tetraethylphosphorodiamidate (5b). 3-Buten-1-ol (1.00 g, 14 mmol) was added dropwise to a stirred solution of phosphorus oxychloride (2.15 g, 14 mmol) in CH_2Cl_2 (60 mL) at 0 °C. The solution was slowly warmed to room temperature and stirred for an additional 8 h. The solution was cooled to 0 °C, and diethylamine (10.24 g, 140 mmol) was added dropwise. The reaction was warmed to room temperature and then heated to reflux for 8 h. The suspension was filtered through diatomaceous earth and the filtrate concentrated. The resulting oil was purified by flash chromatography (EtOAc/hexanes 1:2) to give 5b (2.13 g, 74%) as a clear oil: ^{1}H NMR ($CDCl_3$) 5.82 (1 H, m), 5.15 (2 H, m), 3.94 (2 H, dd), 3.04 (8 H, dq), 2.40 (2 H, m), 1.09 ppm (12 H, t); ^{31}P NMR ($CDCl_3$) −6.63 ppm. Anal. ($C_{12}H_{27}N_2O_2P$) C, H.

3-Butenyl N,N-bis(2-chloroethyl)-N',N'-morpholinophosphorodiamidate (5c) was prepared as described for 5a on a 10-mmol scale, except that morpholine (1.92 g, 22 mmol) was added in place of ammonia and stirred for 8 h. The crude oil was purified by flash chromatography (CH_2Cl_2/acetone 3:1) to give 5c (2.29 g, 66%) as a clear oil: ^{1}H NMR ($CDCl_3$) 5.79 (1 H, m), 5.15 (1 H, m), 4.05 (2 H, m), 3.64 (8 H, m), 3.43 (4 H, m), 3.14 (4 H, m), 2.43 ppm (2 H, m); ^{31}P NMR ($CHCl_3$) −10.35 ppm. Anal. ($C_{12}H_{23}Cl_2N_2O_2P$) C, H.

3-Butenyl Tetrakis(2-chloroethyl)phosphorodiamidate (5d). *n*-Butyllithium (3.65 mL, 5.25 mmol) was added dropwise to 3-buten-1-ol (361 mg, 5.0 mmol) in THF (25 mL) at room temperature, and the solution was stirred for 30 min and then cooled to 0 °C. A solution of phosphoramidic chloride 4b (1.91 g, 5.25 mmol) was added rapidly, the solution was warmed to room temperature, and stirring was continued for 60 min. A solution of KH_2PO_4 (30 mL, 10%) was added and the mixture was extracted with ether (3 × 25 mL). The combined extracts were dried and concentrated to give 5d (1.58 g, 66%) as a pale yellow oil that was used without further purification: ^{1}H NMR ($CDCl_3$) 5.79 (1 H, m), 5.17 (2 H, m), 4.08 (2 H, m), 3.63 (8 H, m), 3.40 (8 H, m), 2.44 ppm (2 H, m); ^{31}P NMR ($CHCl_3$) −9.14 ppm.

2-(4,4,6-Trimethyltetrahydro-1,3-oxazin-2-yl)ethyl N,N-Bis(2-chloroethyl)phosphorodiamidate (8a). A solution of 5a (10.0 g, 36 mmol) in CH_2Cl_2 (150 mL) was cooled to −50 °C, and ozone was bubbled through the solution until a blue color was evident (ca. 20 min). Excess ozone was flushed from the solution with nitrogen, and the ozonide was reduced by the addition of dimethyl sulfide (15.66 g, 252 mmol) at −30 °C. A solution of 4-methyl-4-amino-2-pentanol (8.44 g, 72.0 mmol) in CH_2Cl_2 (20 mL) and anhydrous K_2CO_3 (13.82 g, 100 mmol) was immediately added to the solution. The reaction was warmed to room temperature, and stirring was continued for 3 h. The mixture was filtered, the filtrate concentrated, and the resulting oil purified by flash chromatography (acetone/*t*-BuOH 9:1) to give 5a (2.97 g, 22%) as a 1:1 mixture of diastereomers: ^{1}H NMR ($CDCl_3$) 4.40 (1 H, m), 4.19 (1 H, m), 4.07 (1 H, m), 3.78 (1 H, m), 3.64 (4 H, t), 3.43 (4 H, m), 3.04 (2 H, br s), 1.89 (2 H, m), 1.43 (1 H, m), 1.18 ppm (10 H, m); ^{31}P NMR ($CHCl_3$) −8.42, −8.53 ppm. Anal. ($C_{13}H_{28}Cl_2N_3O_3P$) C, H.

2-(4,4,6-Trimethyltetrahydro-1,3-oxazin-2-yl)ethyl N,N,N',N'-tetraethylphosphorodiamidate (8b) was prepared on a 3,6-mmol scale as described for 8a. The crude product was purified by flash chromatography (acetone/*t*-BuOH 9:1) to give 8b (259 mg, 19%) as a pale yellow oil: ^{1}H NMR ($CDCl_3$) 4.41 (1 H, m), 4.03 (2 H, m), 3.73 (1 H, m), 3.07 (8 H, m), 1.86 (2 H, m), 1.43 (1 H, m), 1.10 ppm (22 H, m); ^{31}P NMR ($CHCl_3$) −6.69 ppm. Anal. ($C_{17}H_{38}N_3O_3P$) C, H.

2-(4,4,6-Trimethyltetrahydro-1,3-oxazin-2-yl)ethyl N,N-bis(2-chloroethyl)-N',N'-morpholinophosphorodiamidate (8c) was prepared on a 1.4-mmol scale as described for 8a. The crude product was purified by flash chromatography (acetone/*t*-BuOH 9:1) to give 8c (259 mg, 19%) as a 1:1 mixture of diastereomers: ^{1}H NMR ($CDCl_3$) 4.38 (1 H, t), 4.13 (2 H, m), 3.76 (1 H, m), 3.61 (8 H, m), 3.37 (4 H, m), 3.15 (4 H, m), 1.88 (2 H, m), 1.44 (1 H, m), 1.13 ppm (10 H, m); ^{31}P NMR ($CHCl_3$) −10.00, −10.05 ppm. Anal. ($C_{17}H_{34}Cl_2N_3O_4P\cdot H_2O$) C, H.

2-(4,4,6-Trimethyltetrahydro-1,3-oxazin-2-yl)ethyl N,N,N',N'-tetrakis(2-chloroethyl)phosphorodiamidate (8d) was prepared on a 1.25-mmol scale as described for 8a. The crude product was purified by flash chromatography (acetone/*t*-BuOH 9:1) to give 8d (210 mg, 33%) as a clear oil: ^{1}H NMR ($CDCl_3$) 4.38 (1 H, m), 4.15 (2 H, m), 3.78 (1 H, m), 3.64 (8 H, m), 3.42 (8 H, m), 1.89 (2 H, m), 1.45 (1 H, m), 1.14 ppm (10 H, m); ^{31}P NMR ($CHCl_3$) −9.06 ppm.

1-Phenyl-2-(4,4,6-trimethyltetrahydro-1,3-oxazin-2-yl)ethyl N,N-Bis(2-chloroethyl)phosphorodiamidate (8e). A solution of 6 (500 mg, 3.55 mmol) in THF (3.6 mL) was cooled to −78 °C, and *n*-butyllithium (2.43 mL, 1.1 equiv) was added over 1 h. After stirring for 1 h at −78 °C, a solution of benzaldehyde (410 mg, 3.9 mmol) in THF (5 mL) was added over 30 min. The reaction mixture was allowed to warm slowly to 0 °C, and a solution of phosphoryl chloride 4a (1.01 g, 3.9 mmol) in 5 mL of THF was added rapidly. After stirring at 0 °C for 15 min, ammonia was bubbled through the reaction mixture for 15 min, and stirring was continued at room temperature for 15 min. The mixture was filtered through diatomaceous earth and the filtrate concentrated.

The crude oxazine 7e was dissolved in THF/ethanol (20 mL, 1:1) and cooled to −35 °C. A solution of sodium borohydride (144 mg, 3.9 mmol) was dissolved in 2 mL of water containing one drop of 40% sodium hydroxide and added dropwise to the solution. The pH was maintained at 6–8 by dropwise addition of 9 N hydrochloric acid, and the temperature was maintained between −35 and −45 °C. After the addition was complete, the reaction mixture was stirred for 1 h. The solution was then poured on ice (10 g) and brought to pH 10 with aqueous sodium hydroxide. The layers were separated, and the aqueous portion was extracted with ether (3 × 20 mL). The combined organic extracts were dried and concentrated; the ^{31}P NMR spectrum of the crude product showed four resonances in a ratio of 46:46:4:4. The crude product was purified by flash chromatography (CH_2Cl_2/acetone 2:3) to give 8e (831 mg, 52%) as a 1:1 mixture of the major diasteromers: ^{1}H NMR ($CDCl_3$) 7.35 (5 H, m), 5.48 (1 H, m), 4.35 (0.5 H, t), 4.25 (0.5 H, t), 3.43 (4 H, m), 3.20 (4 H, m), 2.31 (1 H, m), 1.91

(1 H, m), 1.42 (1 H, m), 1.14 ppm (10 H, m); ^{31}P NMR ($CHCl_3$) -8.94, -9.02 ppm (1:1). Anal. ($C_{19}H_{32}Cl_2N_3O_3P$) H, N; C: calcd 50.54; found: 51.04.

For kinetic studies the two major isomers could be separated by flash chromatography (CH_2Cl_2/acetone 1:1).

1-Phenyl-2-(4,4,6-trimethyltetrahydro-1,3-oxazin-2-yl)ethyl *N,N*-diethylphosphorodiamidate (8f) was prepared as described for **8e**. The crude oil was purified by using flash chromatography (CH_2Cl_2/acetone 1:2) to yield **8f** (353 mg, 34%) as a mixture of diastereomers: ^{1}H NMR ($CDCl_3$) 7.31 (5 H, m), 5.48 (1 H, m), 4.35 (0.5 H, t), 4.25 (0.5 H, t), 3.78 (1 H, m), 2.97 (4 H, m), 2.21 (1 H, m), 1.97 (1 H, m), 1.41 (1 H, m), 1.14 (10 H, m), 0.97 ppm (6 H, t); ^{31}P NMR ($CHCl_3$) -8.88, -9.02 ppm (1:1).

1-Phenyl-2-(4,4,6-trimethyltetrahydro-1,3-oxazin-2-yl)ethyl *N,N*-bis(2-chloroethyl)-*N',N'*-morpholinophosphorodiamidate (8g) was prepared as described for **8e** with the following modifications. Morpholine (5 equiv) was added all at once to the cooled (0 °C) solution in place of ammonia, and the reaction was then stirred for 5 h at room temperature. The crude product was purified by using flash chromatography (CH_2Cl_2/acetone 2:1) to give **5g** (595 mg, 30%) as a 1:1 mixture of diastereomers: ^{1}H NMR ($CDCl_3$) 7.37 (5 H, m), 5.55 (1 H, m), 4.13 (0.5 H, t), 3.93 (0.5 H, dd), 3.68 (5 H, m), 3.32 (4 H, m), 3.17 (4 H, m), 3.03 (4 H, m), 2.27 (1 H, m), 2.02 (1 H, m), 1.41 (1 H, m), 1.11 ppm (10 H, m); ^{31}P NMR ($CHCl_3$) -10.10, -10.28 ppm (1:1).

1-Phenyl-2-(4,4,6-trimethyltetrahydro-1,3-oxazin-2-yl)ethyl *N,N,N',N'*-tetrakis(2-chloroethyl)phosphorodiamidate (8h) was prepared as described for **8e** except that phosphoryl chloride **4b** (1.42 g, 3.9 mmol) was added to the alkoxide at 0 °C. The solution was then heated to reflux for 2 h. Ethanol (30 mL) was added to the cooled solution and the resulting mixture was reduced with sodium borohydride at -40 °C as described above. The crude product was purified by using flash chromatography (EtOAc/hexanes 1:1) to give **5h** (910 mg, 45%) as a 1:1 mixture of diastereomers: ^{1}H NMR ($CDCl_3$) 7.30 (5 H, m), 5.52 (1 H, m), 4.03 (0.5 H, m), 3.80 (0.5 H, m), 3.57 (4 H, m), 3.31 (8 H, m), 2.99 (4 H, m), 2.22 (1 H, m), 2.01 (1 H, m), 1.25 (1 H, m), 1.10 ppm (10 H, m); ^{31}P NMR ($CHCl_3$) -9.37, -9.45 ppm (1:1). Anal. ($C_{23}H_{38}Cl_4N_3O_3P$) H; C: calcd, 40.37; found: 40.87.

Cytotoxicity Assays. Cytotoxic activity was evaluated in vitro by using 1-h drug exposure in a clonogenic assay as previously described.[7] LC_{99} values were obtained by linear regression of log survival vs drug concentration from at least three determinations and are calculated as the drug concentration required to reduce colony formation to 1% of control values. Standard errors are within ±10%. Resistance factors were calculated from the ratio of LC_{99} values for wild-type and cyclophosphamide-resistant cell lines.

Antitumor activity in vivo was determined by using standard NCI protocols for the L1210 line. Tumors were propagated in DBA/2 mice, and drug evaluations were carried out in $B_6D_2F_1$ mice. Mice were randomly assigned to groups of 10 and were injected ip with 10^5 L1210 cells; a suspension of drug in 2% (carboxymethyl)cellulose (0.2 mL) or vehicle alone was injected ip 24 h later. Survivors were counted daily for 30 days. Response is reported as % T/C, calculated as 100 × (treatment survival time)/(control survival time). Survival times of control mice were in the range of 8-10 days for all experiments.

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Registry No. 3d, 135865-43-9; **3e**, 135865-44-0; **4a**, 127-88-8; **4b**, 60106-92-5; **4c**, 1498-54-0; **5a**, 39800-29-8; **5b**, 135865-45-1; **5c**, 135887-43-3; **5d**, 135865-46-2; **6**, 49852-73-5; **7e**, 129904-24-1; **8a** (isomer 1), 135968-84-2; **8a** (isomer 2), 135968-85-3; **8b**, 135865-47-3; **8c** (isomer 1), 135968-86-4; **8c** (isomer 2), 135968-87-5; **8d**, 135865-48-4; **8e** (isomer 1), 135968-88-6; **8e** (isomer 2), 135968-89-7; **8f** (isomer 1), 135969-92-5; **8f** (isomer 2), 135865-49-5; **8g** (isomer 1), 135968-90-0; **8g** (isomer 2), 135968-91-1; **8h** (isomer 1), 135968-92-2; **8h** (isomer 2), 135968-93-3; 4-HC, 39800-16-3; 4-methyl-4-amino-2-pentanol, 4404-98-2; propionaldehyde, 123-38-6; hydrocinnamaldehyde, 104-53-0; bis(2-chloroethyl)amine hydrochloride, 821-48-7; diethylamine, 109-89-7; phosphorus oxychloride, 10025-87-3; 3-buten-1-ol, 627-27-0; benzaldehyde, 100-52-7.

Synthesis and Receptor Binding of Enantiomeric N-Substituted *cis*-*N*-[2-(3,4-Dichlorophenyl)ethyl]-2-(1-pyrrolidinyl)cyclohexylamines as High-Affinity σ Receptor Ligands

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N-Alkyl-substituted derivatives of (+)- and (-)-*cis*-*N*-[2-(3,4-dichlorophenyl)ethyl]-2-(1-pyrrolidinyl)cyclohexylamine have been synthesized in nine steps in a stereospecific manner starting from cyclohexene oxide. The key step in the reaction sequence involved catalytic hydrogenation of oxime 8 in the presence of PtO_2 and AcOH to give the cis diamine (±)-7. Most of the compounds in this series exhibited very high affinity at σ receptors when tested against [^{3}H]-(+)-3-PPP, and in general it was observed that the 1*R*,2*S* enantiomers bound more potently to σ receptors than their corresponding 1*S*,2*R* enantiomers. The most potent σ ligand found in this class was the unsubstituted derivative (1*R*,2*S*)-(-)-4, which exhibited an affinity constant of 0.49 nM. This compound was also found to be very selective for σ receptors. It exhibited little or no affinity for κ opioid, PCP, and dopamine-D_2 receptors. It was also demonstrated that the cis configuration as opposed to the trans configuration of (+)- and (-)-5 was necessary for a higher σ receptor affinity.

Introduction

The σ receptor was originally proposed by Martin et al.[1] based on the ability of the racemic opiate SKF10,047 to produce psychotomimetic effects in dogs. Since that time, σ receptors have undergone several revisions in definition.[2-4] This binding site has been shown to be distinct from either opiate, phencyclidine (PCP), or dopamine-D_2

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