circling studies, only those responding with, at least 200 turns/60 min after apomorphine injection were used. Approximately 40% of the lesioned animals failed this criterion and were discarded. Rotational behavior was evaluated by a rotamer apparatus. The circling behavior was measured as the number of turns performed by the animal every 5 min after apomorphine injection and the extent of circling was recorded for 60 min. Each compound was administered subcutaneously 10 min before apomorphine injection. The results are expressed as the inhibitory dose that produced 50% of inhibition of rotational behavior induced by apomorphine at 60 min.

B.2. Mouse Antinociception Tests. Male mice, weighing 20–30 g, were used. All drugs were dissolved in distilled water or 10% DMSO, a few drops of H_3PO_4, and water. At least three doses were tested, and 6–10 animals per dose were used. ED_{50}'s were calculated by using computerized probit analysis.

B.2.1. Tail-Flick Assays. The procedure and modifications were described (D'Amour and Smith[10] and Dewey et al.[11]) in the literature. Briefly, the mouse's tail was placed in a groove which contained a slit under which was located a photoelectric cell. When the heat source of noxious stimulus was turned on, the heat focused on the tail, and the animal responded by flicking its tail out of the groove. Thus, light passed through the slit and activated the photocell which, in turn, stopped the recording timer. The heat source was adjusted to produce tail flick of 2–4 s under control conditions. Mice were injected sc with drug or vehicle and tested 20 min later. In the assay for antagonism of the analgesic effect, the potential antagonists were administered sc 10 min before the agonist, and evaluation occurred 20 min later.

B.2.2. Phenylquinone Abdominal-Stretching Assay. The procedure was reported previously (Pearl and Harris).[12] The mice were injected sc with drugs and 10 min later received 10.9 μmol/kg ip of a freshly prepared paraphenylquinone (PPQ) solution. The mice were then placed in cages in groups of two each. At 10 min after the PPQ injection, the total number of stretches per group were counted over a 1-min period. A stretch was characterized by an elongation of the mouse's body, development of tension in the abdominal muscles, and extension of the forelimbs. The antinociceptive response was expressed as the percent inhibition of the PPQ-induced stretching response.

B.2.3. Hot-Plate Assay. The method was also reported previously (Eddy and Leimbach[13] and Atwell and Jacobson[14]). The hot plate was held at a constant 55 °C. Mice were placed on the hot plate and activity was scored if the animal jumped or licked its paws after a delay of 5 s or more, but no more than 30 s beyond the control time.

B.2.4. Substitution for Morphine (SDS) in Morphine-Dependent Monkeys. Male and female rhesus monkeys (*Macaca mulatta*) in the weight range of 2.5–7.5 kg were used, and they received 3.9 μmol/kg sc of morphine sulfate every 6 h for at least 90 days. All the animals had received morphine for at least 3 months. Each animal was tested with a new compound with a minimum of 2 weeks recuperation period between tests. At least three monkeys per dose were used. The assay (Aceto et al.)[15,16] was initiated by the subcutaneous injection of the test drug or control substances (morphine and vehicle) into animals in a group that had not received morphine for 14–15 h and showed definite signs of withdrawal. Each animal was randomly allocated to receive one of the following treatments: (a) a dose of the compound under investigation; (b) morphine control, 4.4×10^{-3} mmol/kg; and (c) vehicle control, 1 mL/kg. The animals were scored for suppression of withdrawal signs during a 2.5-h observation period. The observer was "blind" regarding the allocation of treatments. At the end of the study, the data were grouped according to dose and drug. The mean cumulative withdrawal signs ± SEM were calculated and the data illustrated in figure form.

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Aldophosphamide Acetal Diacetate and Structural Analogues: Synthesis and Cytotoxicity Studies[1]

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The synthesis of aldophosphamide acetal diacetate and a number of structural analogues is described. These compounds are designed to undergo biotransformation to the corresponding aldehydes in the presence of carboxylate esterases, enzymes that are ubiquitous in mammalian tissue. Several of these aldehydes can theoretically exist in pseudoequilibrium with the 4-hydroxyoxazaphosphorine tautomers; others lack this capability. The half-lives of the acetals in 0.05 M phosphate buffer, pH 7.4, at 37 °C ranged from 1 to 2 days. In the presence of 2 unit equiv of porcine liver carboxylate esterase, all of the compounds were hydrolyzed with half-lives of less than 1 min. Although closely structurally related, the compounds exhibited a wide range of cytotoxicities to L1210 murine leukemia cells in vitro.

Cyclophosphamide is one of the most extensively used drugs in medical oncology.[2-7] It has a better therapeutic index than most other nitrogen mustards and a much broader spectrum of autitumor activity. Although the principal biotransformation pathways of cyclophosphamide are well-characterized, its mechanism of tumoricidal selectivity remains controversial. Two major viewpoints have emerged on this question. One is that phosphoramide

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(1) (a) Presented in part at the 78th Annual Meeting of the American Association for Cancer Research, Atlanta, GA, May, 1987. Wang, Y.; Newman, R. A.; Farquhar, D. *Proc. Am. Assoc. Cancer Res.* **1987**, *28*, 258. (b) The following abbreviations are used: 4-HC, 4-hydroperoxycyclophosphamide; Mesna, 2-mercaptoethanesulfonic acid sodium salt.
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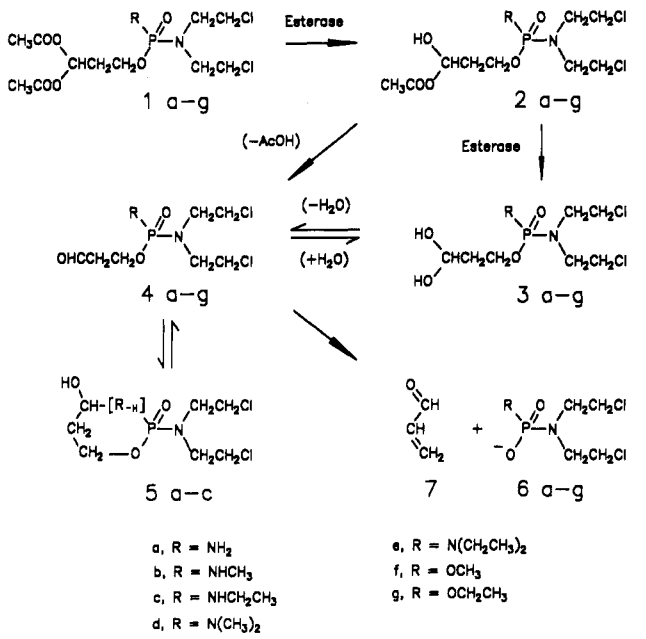
mustard, the ultimate alkylating metabolite,[8] is the principal blood-borne transport form of the drug and is responsible for most, if not all, of its antitumor properties.[9-12] The other is that 4-hydroxycyclophosphamide, the primary oxidative metabolite, and aldophosphamide, its acyclic tautomer, are critical determinants of antitumor selectivity.[13-20] Among the several variants of the latter viewpoint, the selective detoxification hypothesis has commanded much attention. The key tenet of this hypothesis, first proposed by Sladek[13] and later by Conners et al.[14] and Cox et al.,[15] is that the aldehyde dehydrogenase (AlDH) mediated oxidation of aldophosphamide to carboxyphosphamide—a comparatively inert metabolite—is less efficient in drug-sensitive tumor cells than in most normal cells. As a consequence, a relatively greater proportion of aldophosphamide dissociates to the highly cytotoxic phosphorodiamidic mustard in the tumor cells.

Despite its postulated key role in the antitumor selectivity of cyclophosphamide, aldophosphamide has not been definitively synthesized.[21,22] The compound is inherently chemically unstable and dissociates readily to form phosphorodiamidic mustard and acrolein. However, aldophosphamide has been detected spectroscopically as an intermediate in the degradation of 4-hydroxycyclophosphamide[23,24] and mafosfamide (ASTA Z 7557)[25] in aqueous solution. Several stable acyclic derivatives of aldophosphamide have also been reported.[21,26-32] but they

Scheme I



are not known to revert to the parent aldehyde under physiologic conditions.

The major goal of this study was to devise a reliable method to generate aldophosphamide and structurally related compounds from stable precursors under biologic conditions. A secondary goal was to use this method to investigate the structural determinants of cytotoxicity of aldophosphamide. Our approach was to develop chemically stable aldehyde precursors that could be converted in the presence of hydrolytic enzymes to the free parent aldehydes. Carboxylesterase esterases (EC 3.1.1.1, carboxylate ester hydrolases), enzymes that usually show low substrate specificity and are ubiquitous in mammalian tissue,[33] were selected for this purpose. A consideration of the structural and mechanistic requirements for the success of the strategy led to the choice of acetal diacetates of the general structure 1 (Scheme I) as potential aldophosphamide precursors.

The proposed activation mechanism is shown in Scheme I. The parent acetals (1) are expected to be chemically stable under neutral chemical conditions. In the presence of carboxylate esterases, however, one of the carboxylate ester bonds of 1 should be hydrolyzed to generate the corresponding hemiacetal 2. Similar enzymic hydrolysis of the second ester group affords hydrate 3, which should exist in equilibrium with free aldehyde 4. Hemiacetal 2 may also eliminate acetic acid spontaneously to form 4 directly. Once generated, the aldehyde will rapidly equilibrate, if possible, with cyclic tautomer 5. Alterna-

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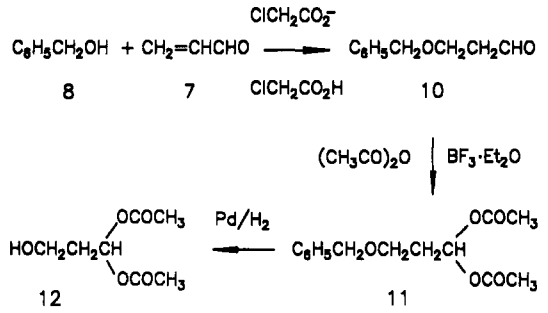
Scheme II



tively, the aldehyde will dissociate to the corresponding phosphoramidate mustard **6** and acrolein (**7**).

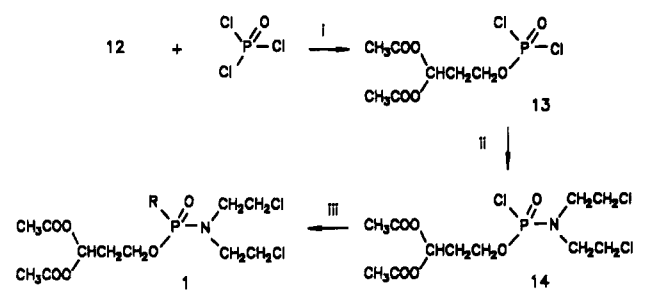
Acetal acetals were selected for two reasons. First, acetate esters are readily cleaved by carboxylate esterases;[33,34] accordingly, the liberated aldehyde hydrates should be formed rapidly. Second, the acetic acid formed should be nontoxic to the host.

To investigate structure–activity relationships for compounds of the general formula **1**, a number of analogues, **1a–g**, were prepared (Scheme I). The R substituents were selected to provide two series of compounds characterized by the ability of the derived aldehydes **4** to form cyclic tautomers. Thus, aldehydes **4a–c** can theoretically exist in equilibrium with the corresponding cyclic tautomers **5a–c** (Scheme I). In contrast, aldehydes **4d–g** cannot cyclize because of the absence of an ionizable hydrogen atom on the R substituent. These latter intermediates are inherently interesting from a mechanistic viewpoint because their inability to form cyclic tautomers should not affect their substrate properties for AlDH. If these "noncyclizable" analogues are differentially metabolized by AlDH in tumor cells and normal cells, as proposed for aldophosphamide, they might possess good antitumor selectivity. On the other hand, if the ability of the aldehyde intermediates to form cyclic tautomers is an important determinant of selectivity, compounds **1d–g** should be less selective than compounds **1a–c**. CH_3O compound **1f** and CH_3CH_2O compound **1g** are isoelectronic with amine analogues **1b** and **1c**; unlike the latter, however, they cannot form cyclic tautomers. In addition to permitting a better understanding of the contribution of cyclic tautomers to the antitumor selectivity of cyclophosphamide, these oxygen isosteres should provide insight into the importance of the non-mustard phosphoramide linkage (i.e., H_2N, CH_3NH, CH_3CH_2NH) to drug selectivity. Limited information is available on this question because substitution of the endocyclic nitrogen atom of cyclophosphamide with heteroatoms such as O and S yields compounds that, apparently,[35] are not bioactivated by mixed-function oxidases.

Chemistry

The most promising approach to the synthesis of aldophosphamide acetal diacetates appeared to be from 3-hydroxypropane-1,1-diyl diacetate (**12**) (Scheme III). Compound **12**, which has not been reported previously, was prepared as shown in Scheme II. Reaction of benzyl alcohol (**8**) with acrolein (**7**) in the presence of $ClCH_2CO_2^-Na^+/ClCH_2CO_2H$ as catalyst[36] gave 3-(benzyloxy)propionaldehyde (**10**). The latter compound reacted rapidly with acetic anhydride in the presence of boron trifluoride/diethyl etherate to give the corresponding acetal diacetate **11**, isolated as a crystalline solid in 75% yield. The identity of **11** was apparent from its NMR spectrum. The aldehyde proton resonance of the precursor (**10**), which appeared as a triplet at δ 9.67, was absent in the spectrum of **11**. It was replaced by a one-proton resonance at δ 6.90 attributable to the acetal methine proton. Hydrogenolysis of **11** over 5% Pd/C in ethyl acetate in the presence of a trace of perchloric acid as catalyst gave 3-hydroxypropane-1,1-diyl diacetate (**12**), quantitatively.

Scheme III



i: Et_3N; ii: $(ClCH_2CH_2)_2NH$, Et_3N; iii: RH, Et_3N

Diacetate derivatives **1a–e** were synthesized as shown in Scheme III. Reaction of alcohol **12** with phosphorus oxychloride in chloroform solution in the presence of triethylamine at −20 °C gave, in situ, phosphorodichloridate **13**, which was converted to phoshoramidochloridate **14** by reaction with bis(2-chloroethyl)amine. The structure of **14** was evident from its NMR spectrum. Thus, the two-proton triplet at δ 3.67 in the spectrum of alcohol **12**, attributable to the terminal methylene hydrogen atoms, now appeared as a complex multiplet at δ 4.43–4.00. The multiplicity of the signal splitting and the approximately 0.7 ppm downfield chemical shift of these resonances clearly indicated that the methylene hydrogens were coupled to the phosphoryl phosphorus atom. Further support for the assigned structure was that the two characteristic four-proton triplets of bis(2-chloroethyl)amine hydrochloride at δ 3.77–3.53 and δ 3.10–2.87, respectively, appeared as a complex eight-proton triplet at δ 3.77–3.20. The latter resonance pattern is typical[23] of phosphoramide mustards. Reaction of phosphoramidochloridate **14** with ammonia in dichloromethane gave **1a**, isolated as a crystalline solid after flash chromatography on silica. Its identity was confirmed from its spectral properties and by elemental analysis. The distinguishing characteristics of the NMR spectrum were a one-proton triplet at δ 6.88 attributable to the acetal methine hydrogen atom, a two-proton multiplet at δ 4.10 attributable to the phosphoryl-bonded methylene group, and a 10-proton multiplet at δ 3.3–3.8 attributable to the bis(2-chloroethyl)amine and amine groups. Phosphorodiamidates **1b–e** were prepared similarly by reaction of **14** with a 2-fold molar excess of the appropriate amine or with 1 molar equiv of the amine hydrochloride in the presence of triethylamine as proton acceptor. The carboxylate ester groups were quite stable under the reaction conditions.

Attempts to prepare phosphoramidates **1f** and **1g** by similar reaction of **14** with the appropriate alcohol were unsuccessful, presumably because of the reduced nucleophilicity of alcohols relative to amines. To avoid harsh chemical conditions that could cleave the carboxylate ester groups, the sequence of reactions, beginning with **13**, was reversed for the preparation of compounds **1f** and **1g**.

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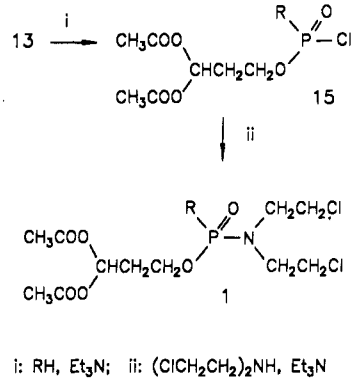
Scheme IV



i: RH, Et_3N; ii: $(ClCH_2CH_2)_2NH$, Et_3N

Table I. Half-Lives of Compounds 1–7[a] in 0.05 M Phosphate Buffer, pH 7.4, at 37 °C, in the Absence or the Presence of Pig Liver Carboxylate Esterase[b]

	half-life			half-life	
no.	buffer only, h	buffer + esterase, s	no.	buffer only, h	buffer + esterase, s
1a	38	36	**1e**	37	40
1b	42	42	**1f**	32	42
1c	40	42	**1g**	29	42
1d	36	38			

[a] The concentration of compounds **1a–g** in solution was 100 μM. [b] Two units of the enzyme per micromole of compound were used. The disappearance of **1a–g** from solution was monitored by HPLC.

Thus, **13** was first reacted with methanol or ethanol to give, in situ, the phosphodiesters **15f** and **15g** (Scheme IV), which were then reacted with bis(2-chloroethyl)amine to give **1f** and **1g**, respectively.

Stability Studies

The stabilities of acetals **1a–g** were determined at a concentration of 100 μM in 0.05 M phosphate buffer, pH 7.4, at 37 °C, either in the absence or the presence of hog liver carboxylate esterase (Table I). The compounds were hydrolyzed slowly in buffer alone; their half-lives ranged from 29 h for **1g** to 42 h for **1b**. In the presence of 2 unit equiv of carboxylate esterase (i.e., two units of the enzyme per micromole of substrate), all of the compounds were degraded in less than 1 min.

Cytotoxicity Studies

The toxicities of the acetals to L1210 leukemia cells during a 1-h incubation at 37 °C are shown in Table II. Cyclophosphamide, 4-HC, mafosfamide, Mesna, phosphorodiamidic mustard (**6**, R = NH_2), and acrolein were tested for comparison. Compound **1a**, with an IC_{50} value of 0.9 μM, was clearly the most toxic member of the series. *N*-Monoalkyl compounds **1b** and **1c**, were 30–40 times less potent. *N,N*-Dialkyl compounds **1d** and **1e** and the *O*-alkyl compounds **1f** and **1g** were relatively weakly toxic; they were 85–270 times less potent than **1a**. Phosphorodiamidic mustard (**6a**), the presumed ultimate alkylating metabolite of **1a**, and acrolein (**7**) were 100 and 50 times less toxic, respectively, than **1a**. In the presence of a 3-fold molar excess of Mesna (a comparatively nontoxic sulfhydryl compound that reacts with acrolein to give a thioether adduct),[37,38] the cytotoxicity of acrolein was decreased more than 3-fold. However, Mesna had no effect on the cytotoxicities of **1a** and 4-HC. The preactivated cyclophosphamide analogues 4-HC and mafosfamide were 40 and 90 times less toxic, respectively, than **1a**.

Table II. Toxicities of Aldophosphamide Analogue Acetals and Related Compounds to L1210 Leukemic Cells in Culture

compd	IC_{50},[a,b] μM	compd	IC_{50},[a,b] μM	compd	IC_{50},[a,b] μM
1a	0.9	**1g**	223	Mesna[e]	>200
1b	38	cyclophos-	>300	acrolein	48
1c	29	phamide		**1a** + Mesna	0.9
1d	>245	4-HC[c]	90	4-HC + Mesna	90
1e	78	mafosfamide	42	acrolein + Mesna	168
1f	150	PM[d]	97		

[a] The drug concentration that reduced the viability of L1210 cells by 50% after 1-h incubation at 37 °C. [b] At least seven drug concentrations ranging from 0.1 to 300 μM were used to determine the IC_{50} values. Each value is the average of duplicate determinations. [c] 4-Hydroperoxycyclophosphamide. [d] Phosphorodiamidic mustard. [e] 2-Mercaptoethanesulfonate disodium salt (an acrolein scavenger).

Discussion

The major objective of this study was to devise a method to generate aldophosphamide and structurally related compounds from stable precursors under biologic conditions that precluded the use of oxazaphosphorines. The study was prompted initially by problems encountered in attempting to extend the mechanistic principles that might contribute to the selectivity of cyclophosphamide to other structural classes of antitumor agents, particularly nucleoside analogues. For example, we previously reported the synthesis of derivatives of 5-fluoro-2′-deoxyuridine[39] and arabinosyl adenine[40] in which a cyclic oxazaphosphorine or dioxophosphorine moiety was present in the 5′-position of the molecule. These cyclic derivatives, in contrast to the parent nucleosides, were only marginally active against murine P388 leukemia in vivo, a finding attributed to their failure to undergo oxidative biotransformation by cytochrome P-450 mixed-function oxidases. As a potential solution to this shortcoming, we considered the preparation of preactivated 5′-cyclic nucleotides, such as 4″-hydroperoxy or 4″-alkylthio derivatives (by analogy with 4-HC and mafosfamide). In practice, however, such compounds are difficult to synthesize. To overcome these problems, we sought a universal means of generating aldophosphamide and structurally related compounds from stable precursors under physiologic conditions that avoided the use of oxazaphosphorines. To explore the feasibility of this approach, aldophosphamide and several structural analogues were selected as model compounds.

The acetal diacetates described here satisfy the research goals. The individual congeners are fairly stable under neutral aqueous conditions, with little difference in inherent stability. Moreover, they are rapidly hydrolyzed in the presence of 2 unit equiv of carboxylate esterase, a finding consistent with the low substrate specificity of the enzyme.[33,41]

Although closely structurally related, the acetals exhibited a broad range of toxicities to L1210 cells. The reasons for these differences remain to be determined;

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(41) The objective of these studies was not to discern small structure-related differences in the rates of enzymic hydrolysis, which would require limiting amounts of the esterase, but to determine whether all of the compounds would be facilely activated in the presence of one or more unit equivalents of the enzyme.

however, they may stem, at least in part, from differences in the abilities of the intermediate aldehydes to form cyclic tautomers, the putative membrane-transport precursors of the cytotoxic ionic phosphoramidate mustards. Thus, of the series of compounds studied, only **1a–c** are capable of giving rise to cyclic intermediates. The most toxic compound, **1a**, has two nitrogen-bonded dissociable hydrogen atoms that can participate in cyclic tautomer formation. Compounds **1b** and **1c**, which were moderately cytotoxic, have only one such hydrogen atom. Compounds **1d–g** cannot form cyclic tautomers; these compounds were only weakly cytotoxic. A further indication that cyclic structures might contribute to the cytocidal potency of aldophosphamide acetals is that *N*-monoalkyl compounds **1b** and **1c** are 5 times more toxic than their oxygen isosteres **1f** and **1g**.

Further studies are required to determine the exact mechanism of bioactivation of these acetals and to account for their marked differences in cytotoxicity. From the data presented here, it cannot be determined whether these acetals are transported unchanged into cells and then hydrolyzed to the corresponding aldehydes or whether they are hydrolyzed extracellularly and taken up as the aldehydes/aldehyde hydrates or the corresponding cyclic tautomers. However, the latter possibility seems more likely because the tissue-culture media contained 10% fetal calf serum, which is rich in carboxylate esterases. It is improbable that extracellularly generated phosphorodiamidic mustard or acrolein contributed significantly to the potency of **1a** since the IC_{50} values of these compounds were 100 and 50 times higher, respectively, than the IC_{50} value of **1a**. Further evidence in support of this interpretation is that Mesna (an acrolein scavenger) had no effect on the cytotoxicity of **1a** but reduced the cytotoxicity of acrolein approximately 3-fold. The increased potency of **1a** relative to those of mafosfamide and 4-HC, either in the presence or absence of Mesna, is also not readily explicable. It may reflect more rapid generation of aldophosphamide/4-hydroxycyclophosphamide from **1a** with greater uptake of the cell-transport form of the drug.

Although the ability of the acetals to generate membrane-transport intermediates may be a critical determinant of their cytocidal potency, other factors may play a role. These include the rates of enzymic hydrolysis of the acetal ester groups, the rates of formation of aldehyde intermediates, the rates of dissociation of the intermediate aldehydes to form alkylating products, the substrate properties of the aldehydes for AlDH, the chemical stabilities of the derived phosphoramide mustards, the sites of interaction of the mustards with DNA, the rates of DNA cross-linking, and DNA repair kinetics. The relative stabilities of the phosphoramide mustards may be particularly important since it has been reported[42] that the presence of electron-donating substituents on the non-mustard nitrogen atom increases the rate of intramolecular O-alkylation; the *mono*alkylating products formed are presumably less cytotoxic than the parent phosphoramide mustards. Structure–activity studies addressing these considerations are in progress.

In summary, a series of chemically stable aldophosphamide analogue precursors have been prepared that are designed to undergo conversion to the corresponding free aldehydes in the presence of carboxylate esterases. Although closely structurally related, the compounds have markedly different biological properties. These differences may relate to their ability to form intermediate cyclic structures. Toxicologic and antitumor screening studies of the acetals are in progress and will be the subject of a future report.

Experimental Section

Chemistry. ^1H NMR spectra were recorded at ambient temperature on a Varian Associates T-60A spectrometer or on an IBM-Bruker Model NR/200 AF spectrometer in the Fourier transform mode, in $CDCl_3$, with tetramethylsilane as an internal standard. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. Melting points were determined on a Hoover capillary apparatus and are uncorrected. All reactions were carried out in dry glassware and were protected from atmospheric moisture. Solvents were dried over freshly activated (300 °C/1 h) molecular sieves (type 4 A). Evaporations were carried out on a rotary evaporator under aspirator vacuum at a bath temperature of <40 °C. The homogeneity of the products was determined by ascending TLC on silica-coated glass plates (silica gel 60 F 254, Merck) using mixtures of $CHCl_3$–acetone (typically 1:1, v/v) or $CHCl_3$–MeOH (typically 4:1, v/v) as the eluting solvent. Chromatograms were visualized under a UV lamp (254 nm) or by placing the air-dried plates in a tank of iodine vapor. To detect alkylating activity, the plates were sprayed with a 1% solution of 4-(*p*-nitrobenzyl)pyridine in acetone and then heated to 100 °C for 5 min and sprayed with a 2% solution of NaOH in EtOH; alkylating compounds appeared as blue spots against a white background. Preparative separations were performed by flash chromatography on silica gel (Merck, 230–400 mesh) with mixtures of $CHCl_3$–acetone or EtOAc–hexane as eluent. All chemical reagents were purchased from Aldrich Chemical Co., Milwaukee, WI.

3-(Benzyloxy)propionaldehyde (10). A solution of benzyl alcohol (123 mL, 128.5 g, 1.19 mol), monochloroacetic acid (6.72 g, 0.071 mol), and NaOH (2.85 g, 0.071 mol) in H_2O (15 mL) was added dropwise, with stirring over 20 min, to acrolein (100 mL, 83.9 g, 1.5 mol) contained in a 500-mL round-bottomed flask. Acetic acid (30 mL, 0.52 mol) was added, and the solution was maintained at 40 °C for 80 h. After being cooled to room temperature, the reaction mixture was washed with H_2O (150 mL × 3), and the organic layer was dried over anhydrous Na_2SO_4. Volatile starting materials and byproducts were removed by vacuum distillation at 100 °C (0.3 mmHg). The viscous oil that remained was aldehyde **10**. Because it appeared homogeneous by TLC [R_f [EtOAc–hexane (1:1)] = 0.77] and NMR analyses, it was used for subsequent reaction without further purification. The yield was 61 g (31%). ^1H NMR ($CDCl_3$): δ 9.67 (t, 1 H, CHO, J_{HH} = 2 Hz), 7.20 (s, 5 H, C_6H_5), 4.43 (s, 2 H, $C_6H_5CH_2$), 3.73 (t, 2 H, OCH_2, J_{HH} = 6 Hz), 2.60 (tt, 2 H, CH_2CHO, J_{HH} = 6 Hz).

3-(Benzyloxy)propane-1,1-diyl Diacetate (11). 3-(Benzyloxy)propionaldehyde (**10**; 40 mL, 44.0 g, 0.27 mol) was added, dropwise, with stirring over 5 min at ambient temperature to a solution of acetic anhydride (40 mL, 0.42 mol), Et_2O (30 mL), and BF_3/Et_2O (3.0 mL) contained in a 500-mL flask. The mixture was stirred for 10 min. It was then washed with 10% NaOAc solution (200 mL) and H_2O (200 mL × 2) and dried over anhydrous Na_2SO_4. The solution was evaporated to give a colorless viscous oil, which crystallized after standing for several days at −13 °C. The product was recrystallized from acetone–hexane. The yield of **11** was 53.9 g (75%). Mp 44–45 °C. ^1H NMR ($CDCl_3$): δ 7.67 (s, 5 H, C_6H_5), 6.90 (t, 1 H, $CH(OAc)_2$, J_{HH} = 6 Hz), 4.47 (s, 2 H, $C_6H_5CH_2$), 3.73 (t, 2 H, OCH_2, J_{HH} = 6 Hz), 2.23–1.90 (m, 2 H, CH_2CH), 2.00 (s, 6 H, 2 CH_3). Anal. ($C_{14}H_{18}O_5$): C, H.

3-Hydroxypropane-1,1-diyl Diacetate (12). A solution of 3-(benzyloxy)propane-1,1-diyl Diacetate (**11**; 1 g, 3.8 mmol) in EtOAc (10 mL) containing 10 μL of 70% perchloric acid was shaken with 5% Pd/C (0.1 g) under an atmosphere of hydrogen at 44 psi for 15 min. The solution was stirred with finely powdered $CaCO_3$ (0.5 g) for 10 min to neutralize the acid and then filtered and evaporated at <30 °C. The residual oil was dried in vacuo (0.01 mmHg) over P_2O_5 at ambient temperature for 48 h. Yield 0.66 g (100%). Because the product was homogeneous by TLC [R_f[EtOAc–hexane (1:1)] = 0.38; R_f [$CHCl_3$–acetone (1:1)] = 0.69] and NMR analyses, it was used for subsequent chemical reactions without further purification. ^1H NMR ($CDCl_3$): δ 6.84 (t, 1 H, $CH(OAc)_2$, J_{HH} = 6 Hz), 4.91 (s, 1 H, OH), 3.67 (t, 2 H, $HOCH_2$,

(42) Engle, T. W.; Zon, G.; Egan, W. *J. Med. Chem.* **1982**, *25*, 1347.

J_{HH} = 6 Hz), 2.16–1.83 (m, 2 H, CH_2CH), 2.06 (s, 6 H, 2 CH_3). Anal. ($C_7H_{12}O_5$): C, H.

3,3-Diacetoxypropyl *N*,*N*-Bis(2-chloroethyl)phosphoramidochloridate (14). A solution of 3-hydroxypropane-1,1-diyl diacetate (12; 2.0 mL, 2.35 g, 14.0 mmol) in CH_2Cl_2 (10 mL) was added simultaneously with a solution of Et_3N (2.0 mL, 14.0 mmol) in CH_2Cl_2 (10 mL), over a period of 30 min, to a stirred solution of $POCl_3$ (1.32 mL, 14.0 mmol) in CH_2Cl_2 (20 mL) maintained at −20 °C in a dry ice–acetone cooling bath. After 20 min, the reaction mixture was warmed to room temperature and stirred for 2 h. Bis(2-chloroethyl)amine hydrochloride (2.52 g, 14.0 mmol) was added, and the mixture was cooled to −20 °C. Et_3N (4.0 mL, 28.0 mmol) was added, dropwise, over 20 min, and the reaction mixture was warmed to room temperature and stirred for a further 2 h. It was then washed with 0.45 M potassium phosphate buffer, pH 7.0 (50 mL), and H_2O (50 mL × 2). The organic phase was dried over anhydrous Na_2SO_4. The oil remaining after evaporation of the solvent was submitted to flash chromatography on a column (5 × 40 cm) of silica (ca. 300 g) using EtOAc–hexane (1:1, v/v) as eluent. Fractions (10 mL each) containing pure **14** as evidenced by TLC analyses [R_f [$CHCl_3$–acetone (1:1)] = 0.58] were combined and evaporated to give **14** as a viscous, pale yellow oil. It was dried in vacuo at 0.01 mmHg over P_2O_5 for 48 h to yield 1.615 g (29%). ^1H NMR ($CDCl_3$): δ 6.83 (t, 1 H, $CH(OAc)_2$, J_{HH} = 6 Hz), 4.43–4.00 (m, 2 H, $POCH_2$), 3.77–3.15 (m, 8 H, 2 CH_2CH_2Cl), 2.37–1.97 (m, 2 H, $CH_2CH(OAc)_2$), 2.07 (s, 6 H, 2 CH_3). Anal. ($C_{11}H_{19}Cl_3NO_6P$): C, H, N.

3,3-Diacetoxypropyl *N*,*N*-Bis(2-chloroethyl)phosphorodiamidate (1a). A solution of 1 N NH_3 in CH_2Cl_2 (12 mL, 12 mmol) was added with stirring over 20 min to a solution of **14** (2.31 g. 5.8 mmol) in CH_2Cl_2 (5 mL) maintained at −20 °C. The mixture was allowed to warm to room temperature and then was stirred for a further 1 h. The solvent was evaporated, and Et_2O (100 mL) was added to the residue. The precipitated salts were filtered off. The solution was evaporated, and the residue was submitted to flash chromatography on silica as described for **14** using $CHCl_3$–acetone (1:1, v/v) as eluent. Fractions containing pure **1a**, as evidenced by TLC [R_f [$CHCl_3$–acetone (1:1)] = 0.5], were combined and evaporated to give a colorless oil, which crystallized on standing at −13 °C. The product was recrystallized from acetone–ether and then dried in vacuo at 0.01 mmHg over P_2O_5 for 48 h to yield 1.57 g (71%). Mp: 51 °C. ^1H NMR ($CDCl_3$): δ 6.88 (t, 1 H, $CH(OAc)_2$, J_{HH} = 6 Hz), 4.10 (m, 2 H, $POCH_2$), 3.8–3.3 (m, 10 H, 2 CH_2CH_2Cl and NH_2), 2.3–2.0 (m, 2 H, $CH_2CH(OAc)_2$), 2.10 (s, 6 H, 2 CH_3). Anal. ($C_{11}H_{21}Cl_2N_2O_6P$): C, H, N.

3,3-Diacetoxypropyl *N*,*N*-Bis(2-chloroethyl)-*N'*-methylphosphorodiamidate (1b). A solution of 3 N $MeNH_2$ in CH_2Cl_2 (3.5 mL, 10.5 mmol) was added with stirring over 20 min to a solution of **14** (2.12 g, 5.3 mmol) in CH_2Cl_2 (10 mL) and maintained at −20 °C. The mixture was stirred for 1 h at room temperature and then worked up as described for **1a**. The product (0.41 g, 20%) was obtained as a colorless oil; it was dried in vacuo over P_2O_5. ^1H NMR ($CDCl_3$): δ 6.86 (t, 1 H, $CH(OAc)_2$, J_{HH} = 6 Hz), 4.23–3.96 (m, 2 H, $POCH_2$), 3.76–3.15 (m, 8 H, 2 CH_2CH_2Cl), 2.78–2.43 (m, 4 H, CH_3NH, J_{PNCH} = 12.6 Hz), 2.18–1.95 (m, 2 H, $CH_2CH(OAc)_2$), 2.08 (s, 6 H, 2 CH_3). Anal. ($C_{12}H_{23}Cl_2N_2O_6P$) C, H, N.

3,3-Diacetoxypropyl *N*,*N*-Bis(2-chloroethyl)-*N'*-ethylphosphorodiamidate (1c). A solution of 3 N $EtNH_2$ in CH_2Cl_2 (3.43 mL, 10.3 mmol) was added, dropwise, with stirring to a solution of **14** (2.05 g, 5.15 mmol) in CH_2Cl_2 (20 mL) at −20 °C. The mixture was stirred for 75 min at room temperature and then worked up as described for **1a**. The product (0.97 g, 46%) was obtained as a colorless oil which crystallized on storage at −13 °C. Mp: 43 °C. ^1H NMR ($CDCl_3$): δ 6.83 (t, 1 H, $CH(OAc)_2$, J_{HH} = 6 Hz), 4.19–3.86 (m, 2 H, $POCH_2$), 3.76–2.70 (m, 11 H, 2 CH_2CH_2Cl and CH_2NH), 2.26–1.93 (m, 2 H, $CH_2CH(OAc)_2$), 2.10 (s, 6 H, 2 CH_3), 1.26–0.98 (m, 3 H, CH_3CH_2NH). Anal. ($C_{13}H_{25}Cl_2N_2O_6P$): C, H, N.

3,3-Diacetoxypropyl *N*,*N*-Bis(2-chloroethyl)-*N'*,*N'*-dimethylphosphorodiamidate (1d). Et_3N (0.93 mL, 6.6 mmol) was added dropwise to a solution of **14** (1.07 g, 2.7 mmol) and Me_2NH·HCl (0.27 g, 3.3 mmol) in CH_2Cl_2 (5 mL) maintained at −20 °C. The mixture was stirred for 2 h at room temperature and then worked up as described for **1a**. The product (0.42 g, 39%) was obtained as a yellow oil. ^1H NMR ($CDCl_3$): δ 6.80 (t, 1 H, $CH(OAc)_2$, J_{HH} = 6 Hz), 4.19–3.86 (m, 2 H, $POCH_2$), 3.70–3.06 (m, 8 H, 2 × CH_2CH_2Cl), 2.73–2.56 (d, 6 H, $(CH_3)_2N$, J_{PNCH} = 12.6 Hz), 2.26–1.96 (m, 2 H, $CH_2CH(OAc)_2$), 2.05 (s, 6 H, 2 CH_3). Anal. ($C_{13}H_{25}Cl_2N_2O_6P$) C, H, N.

3,3-Diacetoxypropyl *N*,*N*-Bis(2-chloroethyl)-*N'*,*N'*-diethylphosphorodiamidate (1e). Et_2NH (0.31 mL, 3.0 mmol) in CH_2Cl_2 (5 mL) was added dropwise to a solution of **14** (0.60 g, 1.51 mmol) in CH_2Cl_2 (10 mL) maintained at −20 °C. The mixture was stirred for 2 h at room temperature and then worked up as described for **1a**. The product (0.24 g, 37%) was obtained as a yellow oil. ^1H NMR ($CDCl_3$): δ 6.86 (t, 1 H, $CH(OAc)_2$, J_{HH} = 6 Hz), 4.20–3.86 (m, 2 H, $POCH_2$), 3.76–2.80 (m, 12 H, 2 CH_2CH_2Cl and 2 CH_3CH_2N), 2.29–1.98 (m, 2 H, $CH_2CH(OAc)_2$), 2.03 (s, 6 H, 2 CH_3), 1.50 (t, 6 H, 2 CH_3CH_2N). Anal. ($C_{15}H_{29}Cl_2N_2O_6P$) C, H, N.

3,3-Diacetoxypropyl Methyl *N*,*N*-Bis(2-chloroethyl)phosphoramidate (1f). A solution of **12** (0.62 g, 3.5 mmol) and Et_3N (0.49 mL, 3.5 mmol) was added dropwise to a solution of $POCl_3$ (0.33 mL, 3.5 mmol) in CH_2Cl_2 (10 mL) maintained at −20 °C. After 20 min, the reaction mixture was allowed to warm to room temperature and was then stirred for another 100 min. It was then cooled to −20 °C, and a solution of MeOH (0.14 mL, 3.5 mmol) and Et_3N (0.49 mL, 3.5 mmol) in CH_2Cl_2 (3 mL) was added. After a further 20 min, the mixture was again allowed to warm to room temperature and was stirred for another 100 min. Bis(2-chloroethyl)amine hydrochloride (0.62 g, 3.5 mmol) and Et_3N (0.98 mL, 7.0 mmol) were added at −20 °C, and the reaction mixture was stirred for a further 2 h at room temperature. The workup procedure was the same as that described for **1a**. The product (0.51 g, 36%) was obtained as a colorless oil. ^1H NMR ($CDCl_3$): δ 6.86 (t, 1 H, $CH(OAc)_2$, J_{HH} = 6 Hz), 4.37–3.30 (m, 13 H, $POCH_2$, CH_3O and 2 CH_2CH_2Cl), 2.26–2.06 (m, 2 H, $CH_2CH(OAc)_2$), 2.03 (s, 6 H, 2 CH_3). Anal. ($C_{12}H_{22}Cl_2NO_7P$): C, H, N.

3,3-Diacetoxypropyl Ethyl *N*,*N*-Bis(2-chloroethyl)phosphoramidate (1g). The procedure was analogous to that described for **1f** except that EtOH (0.27 mL, 4.6 mmol) was used in place of MeOH. The product (0.69 g, 48%) was obtained as a colorless oil. ^1H NMR ($CDCl_3$): δ 6.84 (t, 1 H, $CH(OAc)_2$, J_{HH} = 6 Hz), 4.43–3.26 (m, 12 H, $POCH_2$, CH_3CH_2 and 2 CH_2CH_2Cl), 2.23–2.03 (m, 2 H, $CH_2CH(OAc)_2$), 2.06 (s, 6 H, 2 CH_3), 1.36 (t, 3 H, CH_3CH_2). Anal. ($C_{13}H_{24}Cl_2NO_7P$): C, H, N.

Cyclohexylammonium Hydrogen *N*,*N*-Bis(2-chloroethyl)phosphoramidate. This compound was synthesized according to the published procedure of Friedman and Seligman.[43]

Stability Studies. Porcine liver carboxylate esterase was purchased from Sigma Chemical Co., St. Louis, MO, and was used as received. The specific activity of the preparation was 200 units/mg of protein, where 1 unit is defined as the amount that will hydrolyze 1.0 μmol of ethyl butyrate to butyric acid and ethanol per minute at pH 8.0 at 25 °C. Acetals **1a–g** were dissolved in 0.05 M phosphate buffer, pH 7.4, at a concentration of 100 μM. One-milliliter aliquots of each solution, each contained in a 5.0-mL screw-capped glass vial, were incubated on a shaking water bath at 37 °C in either the absence or the presence of the enzyme. At selected time intervals (typically 2, 4, 8, 12, 16, 20, 30, and 50 h in the absence of enzyme), aliquots (50 μL) were removed and analyzed immediately for parent drug by HPLC on a C-18 reverse-phase column (Waters Associates, Milford, MA; μ-Bondapak C-18; 25 × 4.6 mm, i.d.). A solution of methanol–0.05 M phosphate buffer, pH 6.5 (1:1), at a flow rate of 1.2 mL/min was used as mobile phase. Eluted compounds were monitored with a variable-wavelength UV detector set at 220 nm and 0.01 AUFS sensitivity and quantitated electronically as a function of time using a Hewlett-Packard Model 3390A integrator. The retention times of the parent drugs **1a–g** were 5.9, 6.4, 9.0, 10.2, 13.1, 7.3, and 11.5 min, respectively; the limits of detectability was about 10^{-6} M.

For the enzyme studies, 2 units of esterase per μmol of substrate was used. The enzyme, in 4 μL of solution, was added to 0.2 mL of the drug solution contained in 1.5-mL microcentrifuge tubes

(43) Friedman, O. M.; Seligman, A. M. *J. Am. Chem. Soc.* **1954**, *76*, 655.

that were preequilibrated on a water bath at 37 °C. At intervals of 0.25, 0.5, 1, 2, 3, 4, 5, and 10 min, the vials were removed from the water bath, and 3 volumes (0.6 mL) of cold methanol was added to deactivate the enzyme. The vials were agitated on a Vortex shaker for 20 s and then centrifuged for 4 min at 1000 rpm. Aliquots (50 μL) of the clear supernatant were analyzed by HPLC as described above. In addition to the parent compounds, a progressively increasing peak with a retention time identical with that of acrolein (2.6 min) was present in all of the chromatograms. The half-lives of the parent acetals were determined by linear least-square regression analysis of the pseudo-first-order reactions.

Cytotoxicity Studies. The toxicities of the compounds to L1210 murine leukemia cells were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mossman.[43] The method is dependent on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product that can be measured spectrometrically.

The acetals were dissolved in sterile H_2O to provide stock solutions of 1 mg/mL concentration; these were further diluted with sterile H_2O to afford solutions suitable for the drug concentration range studied. All of the solutions were passed through a 0.22-μm filter (Millipore Corp.) immediately before use.

The L1210 cells were maintained in vitro by serial culture in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, L-glutamine (2 μmol/mL), 2-mercaptoethanol (10 μM), penicillin (50 U/mL), and streptomycin (50 μg/mL) at 37 °C in a humified atmosphere of 5% CO_2 and 95% air; the pH of the medium was 7.4. For the cytotoxicity studies, exponentially growing cells were incubated in the same medium at a concentration of 5×10^5/mL in 75 × 10 mm culture tubes with increasing drug concentrations for 1 h at 37 °C. The cells were washed twice with serum-free medium (2 mL) and harvested by centrifugation for 5 min at 1500 rpm. The cells were resuspended in drug-free medium at a concentration of 1.3×10^5/mL, and 150 μL aliquots (containing approximately 2×10^4 cells) were placed into a 96-well plate and incubated for 72 h at 37 °C. A solution of 75 μg of MTT in 15 μL of sterile H_2O was added to each well, and the plates were incubated for 4 h at 37 °C. Acid-2-propanol (180 μL of 0.04 N HCl in 2-propanol) was then added, and the mixtures were agitated with a pipet to dissolve the crystallized dye. The plates were read on a multiwell scanning spectrophotometer (ELISA reader) at a wavelength of 570 nm. The IC_{50} values were determined by plotting the drug concentration versus the cell viability as described by Mossman.[44] The values in Table II are the average of duplicate determinations.

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Registry No. **1a**, 113341-60-9; **1b**, 123824-17-9; **1c**, 123824-18-0; **1d**, 123824-19-1; **1e**, 123824-20-4; **1f**, 123824-21-5; **1g**, 123824-22-6; **10**, 19790-60-4; **11**, 123824-11-3; **12**, 123824-12-4; **14**, 130197-72-7; $POCl_3$, 10025-87-3; NH_3, 7664-41-7; $MeNH_2$, 74-89-5; $EtNH_2$, 75-04-7; Me_2NH·HCl, 506-59-2; Et_2NH, 109-89-7; MeOH, 67-56-1; EtOH, 64-17-5; cyclohexylammonium hydrogen, 130197-71-6; *N,N*-bis(2-chloroethyl)phosphoramidate carboxylate esterase, 9016-18-6; benzyl alcohol, 100-51-6; acrolein, 107-02-8; bis(2-chloroethyl)amine hydrochloride, 821-48-7.

(44) Mossman, T. J. *Immunol. Methods* **1983**, *65*, 55.

Analogues of Methotrexate and Aminopterin with γ-Methylene and γ-Cyano Substitution of the Glutamate Side Chain: Synthesis and in Vitro Biological Activity[1]

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Analogues of methotrexate (MTX) and aminopterin (AMT) modified at the γ-position of the glutamate side chain were synthesized and evaluated as dihydrofolate reductase (DHFR) inhibitors and tumor cell growth inhibitors. Condensations of 4-amino-4-deoxy-N^{10}-methylpteroic acid (mAPA) with dimethyl DL-4-methyleneglutamate in the presence of diethyl phosphorocyanidate (DEPC) followed by alkaline hydrolysis yielded *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-DL-4-methyleneglutamic acid (γ-methyleneMTX). Condensation of 4-amino-4-deoxy-N^{10}-formylpteroic acid (fAPA) with dimethyl-DL-4-methyleneglutamate by the mixed carboxylic–carbonic anhydride method yielded *N*-(4-amino-4-deoxypteroyl)-DL-4-methyleneglutamic acid (γ-methyleneAMT). Also prepared via DEPC coupling was a mixture of the four possible diastereomers of *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-4-cyanoglutamic acid (γ-cyanoMTX). The requisite intermediate γ-*tert*-butyl α-methyl 4-cyanoglutamate, as a DL-threo/DL-erythro mixture, was prepared from methyl N^{α}-Boc-*O*-tosyl-L-serinate by reaction with sodium *tert*-butyl cyanoacetate followed by mild trifluoroacetic treatment to selectively remove the Boc group. The γ-methylene derivatives of MTX and AMT are attractive because of their potential to act as Michael acceptors within the DHFR active site. γ-CyanoMTX may be viewed as a congener of the nonpolyglutamated MTX analogue γ-fluoroMTX. In vitro bioassay data for the γ-methylene and γ-cyano compounds support the idea that the active site of DHFR, already known for its ability to tolerate modification of the γ-carboxyl group of MTX and AMT, can likewise accommodate substitution on the γ-carbon itself.

Analogues of the classical folic acid antagonists methotrexate (MTX, 1) and aminopterin (AMT, 2) with altered amino acid side chains have been the subject of intensive investigation for over 40 years.[2] Our own studies in this

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(1) Paper 40 in this series; for previous paper, see: Rosowsky, A.; Yu, C.-S. *Pteridines* **1989**, *1*, 143.

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