^{35}S **Enrichment of 3a.** This was done exactly as described above for 1. The recovery of **3a** in the form of **3a′** was 80%, and the specific activity was 41 mCi/mmol.

^{35}S **Enrichment of 3c.** This was done analogously to the method described for 1, with the exception that a different TLC solvent mixture was used, i.e., methylene chloride/ether (1:1). The recovery in the form of **3c′** was 80% with a specific activity of 34 mCi/mmol.

The initial attempts to find a method for ^{35}S enrichment of **3b**, which were analogous to those described above, but under milder conditions, all resulted in the destruction of **3b**.

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Methotrexate Analogues. 15. A Methotrexate Analogue Designed for Active-Site-Directed Irreversible Inactivation of Dihydrofolate Reductase†

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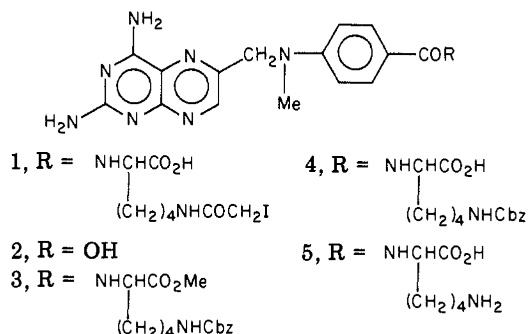
N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-(iodoacetyl)-L-lysine (1) was synthesized as a potential active-site-directed irreversible inhibitor of dihydrofolate reductase (DHFR). In an ultraviolet spectrophotometric assay of dihydrofolate reduction by *Lactobacillus casei* DHFR, 1 and methotrexate (MTX, 4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid) had ID_{50} values of 4.5 and 6.2 nM. The corresponding ID_{50} values in a competitive radioligand binding assay against [^{3}H]MTX were 31 and 16 nM. Thus, as reversible inhibitors of this enzyme over a short exposure time, 1 and MTX had comparable activity. On the other hand, when *L. casei* DHFR was incubated for up to 6 h with 0.1 or 1.0 μM 1, a progressive decrease in the ability of [^{3}H]MTX to subsequently displace the drug was observed. When MTX itself was used at the same concentrations, the extent of displacement of [^{3}H]MTX did not decrease with time. These results were consistent with rapid reversible binding of 1 to the enzyme, followed more slowly by covalent bond formation near the active site. The pH profile for this effect followed a curve with a sigmoidal shape. The apparent inflection point near pH 7.2 was consistent with alkylation of a histidine residue.

The development of a comprehensive model for the interaction of the antitumor agent methotrexate (MTX, 4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid) with its target enzyme dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate:NADPH oxidoreductase EC 1.5.1.3) in intact mammalian cells has been a longstanding goal in a number of laboratories. An important contribution to the understanding of the mechanism of this interaction came from the demonstration that "free" exchangeable MTX in excess of the amount needed to saturate the enzyme and intercept any newly synthesized enzyme is an essential cytotoxic determinant.[1-7] Since even stoichiometrically bound MTX undergoes slow dissociation from the enzyme over time and since even a small fraction (<5%) of the total intracellular DHFR can generate enough tetrahydrofolate to support thymidylate synthesis adquate for cell growth,[6] one reason for seeking to maintain a critical level of free MTX in cells is to prevent this dissociation to the maximum possible extent. It has also been postulated that excess MTX may be necessary to saturate a low-affinity form of the enzyme, possibly associated with inadequate levels of NADPH, that can continue to convert dihydrofolate to tetrahydrofolate even when the high-affinity form is saturated.[3] The existence of low-affinity variants of DHFR in MTX-resistant mammalian cells has in fact been observed in several studies[8-12] and lends support to this concept.

The development of agents that bind more tightly than MTX to both low-affinity and high-affinity forms of DHFR would be a desirable goal in the search for improved folate antagonists.[3] Thus, we became interested in preparing MTX analogues with side-chain functional groups capable of reacting *covalently* with DHFR once the initial reversible enzyme–inhibitor complex has formed. In this paper we report the synthesis of N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-(iodoacetyl)-L-lysine (1), an example



of a "classical antifol" structurally modified with a view

†This paper has been presented in part. See "Abstracts of Papers", 182nd National Meeting of the American Chemical Society, New York, NY, Aug 23–28, 1981, American Chemical Society, Washington, DC, 1981, Abstr MEDI 87.

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to achieving active-site-directed irreversible inactivation of DHFR from *Lactobacillus casei*. Evidence is presented to support the view that this compound probably acts via a mechanism wherein rapid reversible binding to the enzyme is followed more slowly by covalent bond formation near the active site. Beyond its potential utility as an affinity probe for this particular bacterial enzyme, 1 is intended to serve as a model for future studies involving other side-chain analogues of MTX designed for irreversible inactivation of DHFR from mammalian tumors.

Synthesis. 4-Amino-4-deoxy-N^{10}-methylpteroic acid (2)[13] and diethyl phosphorocyanidate[14,15] were condensed at room temperature in dry DMF containing excess Et_3N. The reaction was complete in about 3.5 h as evidence by the appearance of a TLC-mobile spot (R_f 0.61, silica gel, 35:5:1 $CHCl_3$–MeOH–AcOH) which we assume to be the mixed carboxylic diethyl phosphoric anhydride of **2**. Further reaction in situ with *N*-carbobenzoxy-L-lysine methyl ester at room temperature for 3 days yielded N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-carbobenzoxy-L-lysine methyl ester (**3**, 91%). Cleavage of the methyl ester was performed under mild nonracemizing conditions with aqueous piperidine (0–10 °C, 1.5 h) to give the corresponding acid (4, 81%), from which the Cbz group was removed by treatment with boron tribromide in CF_3CO_2H at −20 °C[16] to obtain N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-lysine (**5**, 78%). Alternatively, the Cbz and methyl ester groups were removed from **3** in a single step (91%) by acidolysis with HBr in CF_3CO_2H. In the final step of the synthesis, reaction of **5** with *N*-(iodoacetoxy)succinimide[17] in MeOH at 55–60 °C for 45 min resulted in a 32% yield of **1** after recrystallization. All compounds were characterized on the basis of microanalytical and spectral data, and **1** was shown to have strong alkylating activity in the Epstein test [purple color on reaction with 4-(*p*-nitrobenzyl)pyridine followed by addition of base].[18]

A pivotal aspect of the synthetic sequence was the use of diethyl phosphorocyanidate as the coupling agent. Previous studies in this laboratory have relied on the more traditional mixed carboxylic carbonic anhydride procedure to form amino acid conjugates of **2**.[13,19,20] Diphenylphosphoryl azide has also been investigated, but while it readily converts 1 to an azide derivative, subsequent reaction with amino acid esters either fails or proceeds at a rate too slow to compete with the formation of products arising by Curtius rearrangement.[21,22] The successful synthesis of an amino acid ester adduct from **2** in >90% yield thus represents an important methodologic advance in MTX analogue synthesis.

Enzyme Inhibition. Binding data with DHFR from *L. casei* were obtained for 1, 4, and **5** by means of a competitive radioligand assay using [^{3}H]MTX,[23,24] as well as via a conventional spectrophotometric method based on the change in UV absorbance at 340 nm when NADPH is oxidized to NADP.[24,25] The former procedure gives only a measure of the capacity of a ligand to compete with MTX for the active site, whereas the latter is a functional assay that actually measures the rate of reduction of the normal substrate dihydrofolate. Radioligand binding assays were performed, as well, with a partially purified preparation of DHFR from L1210 mouse leukemia cells. In all instances, MTX was used as a positive control. Since the assays were conducted over a relatively short time span (<15 min), the results represent *reversible* inhibition. It can be seen from Table I that the ratios of ID_{50} values for 1 and MTX against *L. casei* DHFR were 0.7 by spectrophotometric assay and 2.0 by radioligand binding assay. Against DHFR from L1210 cells, the ratio was 3.6. Thus, as a reversible inhibitor of DHFR from either species, **1** was comparable to MTX and was more active than **4** or **5**. The decreased activity of **5** relative to MTX and **1** in both of the bacterial enzyme assays suggests that a positively charged group on the end of the side chain (in this instance, the protonated ϵ-amino group) is detrimental to binding. Neutralization of the positive charge by introduction of a Cbz group seems to counteract this effect. The similar activity of 1 and 4 is consistent with the assumption that initial binding of **1** is reversible and reinforces the view that fairly large structural changes are possible in the γ-terminal region of MTX without significant loss of affinity for the enzyme.[14,26]

Experiments were conducted next to ascertain whether or not 1 can bind *irreversibly* to DHFR from *L. casei*. The three-dimensional structure of this enzyme, as a ternary complex with MTX and NADPH, is known from X-ray crystallographic studies.[27] An examination of the published model shows that the γ-terminal region of the MTX molecule lies in close juxtaposition to the histidine-28 residue of the enzyme. On this basis, we reasoned that the N^{ϵ}-(iodoacetyl) moiety in 1 might well form a covalent

Table I. Reversible Inhibition of *Lactobacillus casei* Dihydrofolate Reductase by N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-(iodoacetyl)-L-lysine (1) and Related Compounds

compd	ID_{50}, nM		
	L. casei [a]	*L. casei* [b]	L1210 [b]
1	4.5	31	9.8
4	14	45	11
5	23	122	11
MTX	6.2	16	2.7

[a] Spectrophotometric assay of conversion of dihydrofolate to tetrahydrofolate;[24,25] ID_{50} = concentration of inhibitor to decrease ΔOD_{340} by 50%. Results are means of triplicate assays (±10%). [b] Competitive [^{3}H]MTX binding assay of affinity for DHFR;[23,24] ID_{50} = concentration of inhibitor to decrease binding of radioligand by 50%. Results are means of triplicate assays (±10%).

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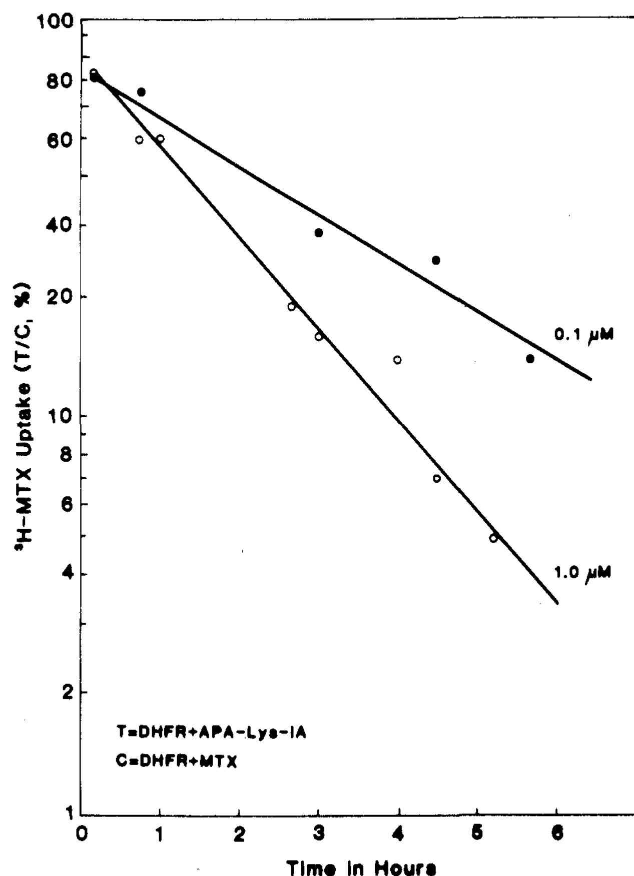

Figure 1. Decreased binding of [^{3}H]MTX to *L. casei* DHFR following incubation with compound 1.

bond to one of the imidazole nitrogens in this particular residue.

A modification of the method developed by Cohen and co-workers[28] to measure displacement of [^{3}H]MTX from L1210 DHFR by unlabeled MTX was employed to demonstrate time-dependent inactivation of DHFR by 1. Trial experiments indicated that the rate of dissociation of the reversible enzyme–inhibitor complex (i.e., the "off rate") was slow, requiring long incubation with excess [^{3}H]MTX to assure complete displacement. The ability of *L. casei* DHFR to bind [^{3}H]MTX following exposure to two concentrations of 1 (0.1 and 1.0 μM) for up to 6 h at pH 7.4, expressed as a percentage of MTX-treated controls (% T/C), is shown in Figure 1. The time required to elicit a 50% decrease in T/C for [^{3}H]MTX binding at concentrations of 0.1 and 1.0 μM was calculated from the slopes of the lines to be 141 ± 27 and 82 ± 18 min, respectively. The ability of the enzyme to bind [^{3}H]MTX after exposure

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(29) Compound 5 was obtained previously[30] in low overall yield via a three-component synthesis starting from N^{α}-[*p*-(methylamino)benzoyl]-N^{ϵ}-carbobenzoxy-L-lysine methyl ester, 2,4,5,6-tetraaminopyrimidine, and 2,3-dibromopropionaldehyde (Waller Reaction). Following deprotection with HBr in CF_3CO_2H, the product was isolated as a hydrated trihydrobromide. Subsequent to submission of the present paper, another publication[31] reported a synthesis of 5 (<15% overall yield) from 2 and N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-carbobenzoxy-L-lysine *tert*-butyl ester by conventional mixed carboxylic carbonic anhydride coupling and deprotection with boron tribromide in CF_3CO_2H. In this case, the product was isolated as a hydrated hemihydrobromide. Insofar as we know, the free acid-free form of 5 has not been reported until now.

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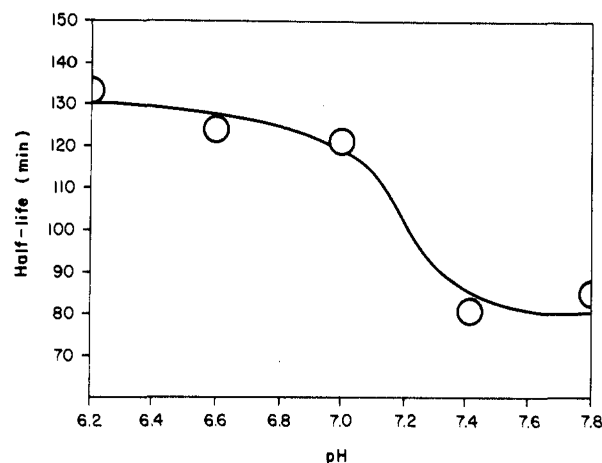

Figure 2. pH dependence for the irreversible inactivation of *L. casei* DHFR by compound 1.

to 0.1 μM 1 for 5.5 h, followed by a 24-h exchange period in the presence of [^{3}H]MTX as described under Experimental Section, fell to about 15% of the MTX-treated controls. After a similar 5.5-h treatment with 1.0 μM 1, the extent of ligand displacement relative to MTX-treated controls was only 4%. It should be noted that extrapolation of the dose–response curve to zero time does not intersect the ordinate at 100%. We believe this reflects the fact that, since the "off rate" of the noncovalently bound inhibitor is slow, some covalent modification continues to occur on the enzyme during the displacement step by [^{3}H]MTX. The extent of this reaction appears to be ~15% as judged from the finding that the extrapolated intercept for the two concentrations of 1 is between 80 and 90%. When the pH profile for inactivation was examined (Figure 2), a curve with a sigmoidal shape was obtained. The estimated inflection point of pH 7.2 corresponded rather well to the pK_a for an imidazole nitrogen in histidine. These results were consistent with a mechanism wherein rapid, reversible binding of 1 to DHFR is followed more slowly by *irreversible covalent linkage* to a nonprotonated nitrogen on a histidine residue near the active site. An alternative possibility that cannot be ruled out at this time is that alkylation involves reaction with another amino acid residue whose pK_a in the microenvironment of the enzyme is around 7.2. Further studies utilizing radioactively labeled 1, which are presently in progress, are expected to provide a more definitive answer to this question.

Current work in our laboratory is directed toward the synthesis of other MTX analogues specifically designed as irreversible inhibitors of DHFR from mammalian cells, as opposed to *L. casei*. These studies should be greatly stimulated as X-ray crystallographic information about the three-dimensional structure of these enzymes becomes available.

Experimental Section

Melting points were determined in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, MA) and are uncorrected. NMR spectra were recorded on a Varian T60A instrument with Me_4Si as the reference, and UV absorbance was measured by means of a Cary Model 15 spectrophotometer. Unless otherwise specified, column chromatography was performed on 100–200 mesh silica gel (Fisher Scientific Co., Boston, MA), and TLC analyses were carried out on Analtech GHLF silica gel plates (0.25 mm), which were visualized under UV light at 254 nm. Microchemical analyses were performed by Galbraith Laboratories, Knoxville, TN. N^{ϵ}-Carbobenzoxy-L-lysine methyl ester hydrochloride was purchased from Chemical Dynamics, South Plainfield, NJ. [3′,5′,7-^{3}H]MTX (sp act. 18.1 Ci/mmol),

hereafter referred to as [^{3}H]MTX, was obtained from Amersham, Arlington Heights, IL. DHFR from MTX-resistant *L. casei* and carboxypeptidase G_1 (CPG_1) from *Pseudomonas sp.* were provided by the New England Enzyme Center, Boston, MA, and were kept frozen at -20 °C until use. The CPG_1 was supplied in 50 mM Tris-HCl, pH 7.3, containing 10 μM $ZnCl_2$ and had a specific activity of 3200 units/mL. Bovine serum albumin type V (BSA), neutral activated charcoal, swine gelatin type I, dextran (M_r 161000), and NADPH were purchased from Sigma Chemical Co., St. Louis, MO. Competitive ligand-binding assays were carried out in rigid polystyrene microtiter plates containing 8 × 12 U-shaped wells (Dynatech, Arlington, VA). Scintillation counting was performed in Biofluor (New England Nuclear, Boston, MA) using a Beckman Model LS-7000 instrument with a counting efficiency of 41%.

4-Amino-4-deoxy-N^{10}-methylpteroic Acid Dihydrate (2). An amount of MTX sodium salt equivalent to 5 g of free acid was dissolved in 2 L of 1 N NaOAc containing 200 mg of dissolved $ZnCl_2$, and 100 μL of freshly thawed CPG_1 solution was added. The mixture was incubated at 37 °C overnight, then cooled to 5 °C, and adjusted to pH 6 with AcOH. The precipitate was suction filtered, washed with ice-water (10 mL), and dried in vacuo at 45 °C for 4 h: yield 3.58 g (99%). Since the product was hygroscopic, it was left exposed to ambient laboratory air until it reached constant weight before being sent out for microanalysis. We have found that material prepared *precisely* in this way is much more soluble in DMF than the hydrated HCl salt described previously[13] and gives superior yields in coupling reactions with amino esters using the diethyl phosphorocyanidate procedure.[14] Anal. ($C_{15}H_{15}N_7O_2{\cdot}2.08H_2O$) C, H, N.

N^{α}-(Amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-carbobenzoxy-L-lysine Methyl Ester (3). To a solution of 2 (2.00 g, 0.00553 mol) in dry DMF (600 mL) were added Et_3N (2 mL) and diethyl phosphorocyanidate (2.24 g, 0.0115 mol),[15] and stirring was continued at room temperature for 3.5 h. The mixture was then added to a rapidly stirred solution of N^{ϵ}-carbobenzoxy-L-lysine methyl ester hydrochloride (3.00 g, 0.00907 mol) in dry DMF (50 mL). After 3 days at room temperature, the solvent was removed with the aid of a rotary evaporator (60–65 °C bath). The viscous residue was dissolved in 10:1 $CHCl_3$–MeOH (50 mL), and the solution was applied onto a column of silica gel (2.6 × 35 cm), which was eluted successively with the same solvent mixture (1.2 L) and then with 6:1 $CHCl_3$–MeOH (1.2 L). Volumes of 7.5 mL were collected into individual tubes, which were monitored by TLC (silica gel, 35:15:1 $CHCl_3$–MeOH–AcOH). The first 100 mL, containing some TLC-mobile impurities, was discarded. Appropriate TLC-homogeneous fractions of the subsequent eluent were pooled and evaporated. The residue was dissolved in 10:1 $CHCl_3$–MeOH (180 mL), and this solution was slowly poured into vigorously stirred Et_2O (1 L) to form a yellow precipitate, which was filtered and dried. The product was triturated with distilled H_2O (120 mL) with the aid of an ultrasonic bath to remove a trace of remaining N^{ϵ}-carbobenzoxy-L-lysine methyl ester (TLC-detectable by ninhydrin spray), filtered, washed again with H_2O (10 mL), and dried in vacuo overnight at room temperature: yield 3.13 g (91%); mp 131–133 °C; NMR (CF_3CO_2H) δ 9.00 (s, 1 H, CH=N), 8.10 (m, 4 H, C_6H_4CO), 7.42 (m, 5 H, C_6H_5), 5.48 (s, 2 H, CH_2N), 5.30 (s, 2 H, CO_2CH_2), 4.97 (m, 1 H, $CHCO_2$), 3.98 (s, 3 H, CO_2CH_3), 3.75 (s, 3 H, NCH_3), 3.37 (s, 2 H, CH_2NHCbz), 1.26–2.50 [m, 6 H, $(CH_2)_3$]. Anal. ($C_{30}H_{35}N_9O_5{\cdot}H_2O$) C, H, N.

N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-carbobenzoxy-L-lysine (4). Crushed ice (200 g) was added over 40 min to a vigorously stirred solution of the ester 3 (1.5 g, 0.0024 mol) in ice-cold piperidine (55 mL). Stirring at 0–10 °C was continued for another 1.5 h, the piperidine and H_2O were removed under reduced pressure, and the residue was redissolved in H_2O (200 mL). The pH was adjusted to 5.5 with 0.1 N HCl, and after overnight storage at 0–5 °C, the precipitate was filtered, washed with ice-water (20 mL), and dried in vacuo at 35 °C for 5 h: yield 1.20 g (84%); mp 182–184 °C dec; NMR (CF_3CO_2H) δ 8.98 (s, 1 H, CH=N), 8.08 (m, 4 H, C_6H_4CO), 7.42 (m, 5 H, C_6H_5), 5.43 (s, 2 H, CH_2N), 5.27 (s, 2 H, CO_2CH_2), 4.97 (m, 1 H, $CHCO_2$), 3.73 (s, 3 H, NCH_3), 3.37 (s, 2 H, CH_2NHCbz), 1.24–2.46 [m, 6 H, $(CH_2)_3$]. Anal. ($C_{29}H_{35}N_9O_5{\cdot}3H_2O$) C, H, N.

N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-L-lysine (5). Method A. Boron tribromide (2.5 g, 0.01 mol) was added dropwise to a vigorously stirred solution of the Cbz derivative 4 (1.0 g, 0.00156 mol) in CF_3CO_2H (10 mL) at -20 °C.[16] Addition was conveniently carried out by placing an inverted 5-mL ampule of the prefrozen boron tribromide into the neck of a ground-glass reducing adapter that was connected at its narrow end to the reaction flask and at the other end to an L-shaped venting tube. When most of the boron tribromide had melted into the reaction vessel, vigorous effervescence occurred. After gas evolution ceased, the venting tube was removed, and the mixture was stirred under a slow stream of dry N_2 for 1 h. The solvent was then evaporated under reduced pressure, and the residue was dissolved in 2 N NH_4OH and chromatographed in 12 batches of 5 mL each on a column of Sephadex CM-25 (Pharmacia, NH_4^+ form). Deionized H_2O was used for elution, and TLC-homogeneous fractions were pooled appropriately, concentrated to a volume of 100 mL by rotary evaporation, and freeze-dried: total yield 0.58 g (78%); mp 228–232 °C (darkening above 204 °C); TLC R_f 0.34 (silica gel, 3:1:1 *n*-BuOH–AcOH–H_2O); UV (0.1 N NaOH) 257 nm (ϵ 26000), 302 (25000), 370 (8100); UV (0.1 N HCl) 244 nm (ϵ 19000), 307 (22000); MS (field desorption), *m/e* 453 (M^+); NMR (CF_3CO_2H) δ 8.93 (s, 1 H, CH=N), 8.02 (m, 4 H, C_6H_4CO), 5.40 (s, 2 H, CH_2N), 4.90 (s, 1 H, $CHCO_2$), 3.67 (s, 3 H, NCH_3), 3.03 (s, 2 H, $CH_2NH_3^+$), 1.0–2.6 [m, 6 H, $(CH_2)_3$]. Anal. ($C_{21}H_{27}N_9O_3{\cdot}3H_2O$) C, H, N.[29]

Method B. In an alternative procedure that gave a higher overall yield, the Cbz ester 3 (0.500 g, 0.985 mmol) was dissolved in anhydrous CF_3CO_2H (5 mL) under a dry N_2 atmosphere at room temperature. Anhydrous HBr gas was bubbled through the solution for 5 min. The gas delivery tube was then replaced with a condenser cooled by ice-cold H_2O, and the solution was refluxed for 5 min until a slight darkening occurred. After being cooled to room temperature, the reaction mixture was evaporated to dryness under a stream of dry N_2. The viscous residue was triturated with Et_2O (45 mL) in an ultrasonic bath to obtain a solid, which was filtered, redissolved in H_2O (5 mL), and chromatographed on Sephadex CM-50 (Pharmacia, NH_4^+ form, 2.6 × 65 cm). The column was eluted successively with deionized H_2O (200 mL) and 0.3 M $(NH_4)_2CO_3$ (400 mL). Appropriate TLC-homogeneous fractions were pooled and freeze-dried: yield 0.374 g (91%). The product obtained in this manner was indistinguishable from the material prepared via method A.

N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-(iodoacetyl)-L-lysine (1). Anhydrous MeOH (10 mL) was added to a septum-stoppered glass pressure bottle containing 5 (44.5 mg, 0.087 mmol) and *N*-(iodoacetoxy)succinimide (60 mg, 0.23 mmol),[17] and the mixture was heated to 55–60 °C (bath temperature) for 45 min. The clear solution was poured into Et_2O (80 mL), and the precipitate was filtered, washed with Et_2O, and redissolved in 10:1 MeOH–AcOH (20 mL) at the boiling point. The hot solution was filtered into EtOAc (80 mL), and the mixture stored in freezer for 2 days. The product that crystallized out was filtered, washed with Et_2O (10 mL), and dried in vacuo: yield 19.8 mg (32%); mp 175–180 °C dec (softening at 120 °C); TLC R_f 0.48 (silica gel, 3:1:1 *n*-BuOH–AcOH–H_2O). Anal. ($C_{23}H_{28}N_9O_4I{\cdot}H_2O$) C, H, N, I.

Treatment of this compound with 10% 4-(*p*-nitrobenzyl)pyridine in refluxing acetone, followed by cooling to 25 °C and addition of Et_3N, gave an intense purple color indicative of alkylating activity (positive Epstein test).[18] A 0.155 mM stock solution of 1 in deionized H_2O containing a precise stoichiometric equivalent of $NaHCO_3$ was prepared for enzyme assays. The solution was stored in a glass container at -20 °C in the dark for up to 6 months without discernible loss of alkylating activity.

Time-Dependent Inactivation of *Lactobacillus casei* DHFR. The ability of 1 to compete with [^{3}H]MTX for binding to *L. casei* enzyme was measured via a modification of the method used previously by others[28] to study [^{3}H]MTX displacement from L1210 enzyme by unlabeled MTX. [^{3}H]MTX (sp act. 18.1 Ci/mmol) was purified by column chromatography on DEAE-cellulose[32] and made up to a concentration of 55 nM in 0.1 M KH_2PO_4 buffer, pH 7.4. This solution gave 8.8 × 10^4 cpm/100

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μL on scintillation counting. The enzyme was made up in 0.1 M KH_2PO_4 buffer, pH 6.2, containing 0.1% BSA and 1 mM NADPH, and this stock solution was divided into 0.5-mL aliquots, which were kept frozen at -20 °C until use. The charcoal mixture used to remove nonbound ligand consisted of 10 g of prewashed neutral activated charcoal, 2.5 g of BSA, and 0.1 g of dextran in 100 mL of 0.1 M KH_2PO_4 buffer, pH 7.4, containing 0.1% swine gelatin. The charcoal was washed initially with three portions of the pH 7.4 buffer to remove the "fines" and then twice more with buffer containing 0.1% gelatin. All reagents were made up fresh with deionized H_2O and kept cold during use.

In a typical experiment, freshly thawed enzyme was diluted 250-fold with pH 7.4 buffer containing 2 mM NADPH. At this dilution, the enzyme had the capacity to bind approximately 10% of the added counts of [^{3}H]MTX under the conditions described below. A suitable amount of diluted enzyme (usually 0.8–1.0 mL) was added to a 15-mL Falcon plastic tube containing an equal volume of either 200 nM test compound (1 or MTX) or control buffer. The assay mixture was incubated in the dark at 37 °C, and at each time point a 200-μL aliquot was transferred to one of the wells of a cold microtiter plate (ice bath). A 67-μL aliquot of ice-cold charcoal mixture was then pipetted into each well to remove excess nonbound ligand, and the contents of the well were mixed thoroughly with the aid of the pipet. To ensure reproducibility in this step and the subsequent charcoal treatment (see below), the contents of the well were always *drawn up and released exactly five times*. After centrifugation at 100*g* (10 min, 2 °C), the supernatant in each well was removed and immediately dispensed in triplicate 67-μL portions to a second microtiter plate, each well of which contained 100 μL of [^{3}H]MTX and 1 mM NADPH. After 24 h at room temperature to allow displacement of reversibly bound drug by [^{3}H]MTX, the plate was cooled on ice for 5 min, and 50 μL of cold charcoal mixture was added to remove excess nonbound [^{3}H]MTX. As in the first charcoal treatment, repeated pipetting (5 times) of the contents of each well was performed to ensure good mixing. After a second centrifugation at 1000*g* (10 min, 2 °C), 108 μL of the supernatant was added to a vial containing 5 mL of Biofluor, and the amount of enzyme-bound radioactivity was determined by scintillation counting. Appropriate background corrections were made with the aid of a blank, and results were expressed as % T/C values from the following equation (see Figure 1 for results):

$$\% \text{ T/C} = \frac{\text{DHFR-bound [}^3\text{H]MTX (cpm) after treatment with 1}}{\text{DHFR-bound [}^3\text{H]MTX (cpm) after treatment with MTX}}$$

The assay described above was also performed at several pH values ranging from 6.2 to 7.8 to generate a pH profile for the time-dependent inactivation of the enzyme by 1. The inhibitor concentration in this series of experiments was 1 μM. The results are presented in Figure 2.

In a control experiment using iodoacetamide as the inhibitor, no time-dependent incactivation of enzyme was observed.

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Antimalarials. 13. 5-Alkoxy Analogues of 4-Methylprimaquine

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A series of nuclear and side-chain analogues of 4-methylprimaquine incorporating an alkoxy group in the 5-position of the quinoline nucleus has been prepared. The compounds were tested for suppressive antimalarial activity against *Plasmodium berghei* in mice and for radical curative antimalarial activity against *Plasmodium cynomolgi* in the rhesus monkey. Although the toxicity problems characteristic of the 8-aminoquinolines were not overcome, several of the compounds, surprisingly, were highly effective as both blood and tissue schizonticidal agents.

In an earlier paper[1] in this series, we reported the preparations of several 4-substituted primaquine analogues. Such a study was undertaken based on the fact that 4-methylprimaquine[2a,b] was approximately twice as effective as primaquine itself against exoerythrocytic forms of *Plasmodium cynomolgi* in the rhesus monkey.[3] Additionally, the compound was slightly less toxic and slightly more effective than primaquine as a blood schizonticide in the *Plasmodium berghei* Rane mouse screen. However, none of the other 4-substituted primaquine analogues prepared by us possessed appreciable activity in either of these screens.

The present investigation was undertaken in view of findings that 5-methoxypentaquine and related 5,6-disubstituted 8-aminoquinolines possessed higher therapeutic indexes relative to the corresponding 5-unsubstituted analogues.[4]

A logical course to pursue, therefore, was to prepare 5-alkoxy analogues of 4-methylprimaquine. It was felt that the presence of a 4-methyl substituent would both enhance antimalarial activity and reduce toxicity as was the case in the primaquine series. Also, it was of interest to prepare related bridged 5,6-alkylenedioxy analogues in order to compare their efficacies with those of the acyclic analogues.

Chemistry. The synthetic sequence shown in Scheme I is representative of that used to prepare the 11 target compounds reported herein and is essentially identical with that described earlier[1] to prepare a series of 4-substituted primaquine analogues.

The three requisite substituted nitroanilines have been reported. 4-Amino-5-nitroveratrole was prepared by Drake et al.,[5] and 4-amino-5-nitro-1,4-benzodioxane and 4-

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