$^{14}CO_2$ released (dpm) with L-carnitine; B_i, $^{14}CO_2$ released (dpm) without L-carnitine.

Acknowledgment. We are grateful to Drs. K. Morita, M. Yoneda, and Y. Nakao for their interest throughout this work. Thanks are also due to the members of physical analysis and large scale production sections for their support and to H. Fujiuchi and K. Kawakami for their assistance.

Methotrexate Analogues. 30. Dihydrofolate Reductase Inhibition and in Vitro Tumor Cell Growth Inhibition by N^{ϵ}-(Haloacetyl)-L-lysine and N^{δ}-(Haloacetyl)-L-ornithine Analogues and an Acivicin Analogue of Methotrexate[1]

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Analogues of methotrexate (MTX) with strong alkylating activity were prepared by replacing the L-glutamate side chain with N^{ω}-haloacetyl derivatives of L-lysine and L-ornithine. Haloacetylation was accomplished in 30–40% yield by reaction of the preformed L-lysine and L-ornithine analogues of MTX with *p*-nitrophenyl bromoacetate or chloroacetate in aqueous sodium bicarbonate at room temperature. All four haloacetamides were potent inhibitors in spectrophotometric assays measuring noncovalent binding to purified dihydrofolate reductase (DHFR) from L1210 cells. In experiments designed to measure time-dependent inactivation of DHFR from L1210 cells and *Candida albicans*, the N^{ϵ}-(bromoacetyl)-L-lysine and N^{δ}-(bromoacetyl)-L-ornithine analogues gave results consistent with covalent binding, whereas N^{ϵ}- and N^{δ}-chloroacetyl analogues did not. The N^{δ}-(bromoacetyl)-L-ornithine analogue appeared to be the more reactive one toward both enzymes. Amino acid analysis of acid hydrolysates of the L1210 enzyme following incubation with the bromoacetamides failed to demonstrate the presence of a carboxymethylated residue, suggesting that alkylation had perhaps formed an acid-labile bond. In growth inhibition assays with L1210 cultured murine leukemia cells, the four haloacetamides were all more potent than their nonacylated precursors but less potent than MTX. The >40 000-fold MTX-resistant mutant cell line L1210/R81 was only partly cross-resistant to the haloacetamides. An analogue of MTX with acivicin replacing glutamate was a potent inhibitor of DHFR from chicken liver and L1210 cells but was 200 times less potent than MTX against L1210 cells in culture.

We have reported previously[2] that N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-(iodoacetyl)-L-lysine (**1**) is an active-site-directed irreversible inhibitor of dihydrofolate reductase (DHFR), the primary enzyme target for methotrexate (MTX) and other antifolates of the 2,4-diaminopyrimidine type.[3] In spectrophotometric and competitive ligand binding assays of reversible binding to bacterial (*Lactobacillus casei*) and mammalian (murine L1210 leukemia) DHFR, **1** showed inhibition comparable to that of MTX. Moreover, when **1** was incubated with DHFR from *L. casei* for several hours, there was a progressive loss of ability to bind MTX, which was consistent with covalent reaction at the active site. From a consideration of the known three-dimensional structure for the *L. casei* ternary DHFR–MTX–NADPH complex[4] and from the finding that the pH profile for inactivation by **1** had an inflection between 7.0 and 7.4, it was surmised that covalent reaction was occurring at His-28.[2] Subsequent work confirmed this and revealed that **1** can react with either of the imidazole nitrogens of His-28, suggesting that the imidazole ring may be capable of free rotation when the ternary enzyme complex is in solution.[5] It was also noted that **1** can bind covalently to DHFR from chicken liver and from MTX-resistant human lymphoblasts (WI-L2/M4 cells), apparently by alkylation of cysteine. This was most surprising, since the single cysteine (Cys) residue in these enzymes is believed to lie outside the active site, where it cannot easily be reached without a major conformational change in protein secondary structure. These novel findings prompted us to undertake a study of analogues of **1** with bromoacetyl or chloroacetyl groups in place of iodoacetyl and with the L-lysine moiety replaced by α,ω-diaminoalkanoic acids containing fewer CH_2 groups. This paper describes the hitherto unknown L-lysine analogues **2** and **3** and the L-ornithine analogues **4** and **5**. In addition we report the preparation and biological activity of the analogue **6**, in which the glutamate moiety is replaced by L-3-chloro-4,5-dihydro-5-isoxazoleacetate (acivicin). Interest in **6** stemmed from a desire to introduce into the molecule other amino acids with a demonstrated ability to react covalently with enzymes. Irreversible inactivation of L-glutamine synthetase by acivicin is well-known and has been ascribed to electrophilic attack of a thiol group.[6] Because of the chemical

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(1) Paper 29: Rosowsky, A.; Forsch, R. A.; Freisheim, J. H.; Danenberg, P. V.; Moran, R. G.; Wick, M. M. *J. Med. Chem.* **1986**, *29*, 1872.
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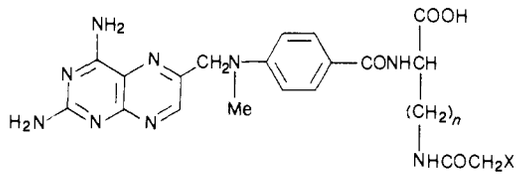
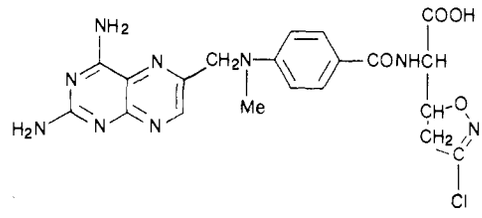


1-3: $n = 4$; X = I, Br, Cl
4,5: $n = 3$; X = Br, Cl



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reactivity of its 3-chloro substituent, acivicin may thus be viewed broadly as an affinity label.[7,8]

Chemistry. As others have noted,[9] the preparation of N^{ω}-haloacetyl derivatives of compounds containing an aliphatic primary amino group and a 2,4-diaminopyrimidine moiety can be difficult. In our reported synthesis of 1, N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-lysine was acylated with *N*-(iodoacetoxy)succinimide.[2] The yield was only 32%, and we therefore sought to increase it by varying the acylating agent, reaction solvent, and method of isolation. After considerable experimentation, it became clear that standard acylation techniques using haloacetyl halides or haloacetic anhydrides would not succeed and that milder reagents should be used with these sensitive compounds. While *N*-(haloacetoxy)succinimide reacted satisfactorily, we considered *p*-nitrophenyl haloacetates to be more attractive because the disappearance of the UV-absorbing acylating agent and concomitant formation of *p*-nitrophenol could be monitored by TLC. The product was readily detected with the aid of the alkylation reagent (*p*-nitrobenzyl)pyridine/Et_3N.[10] Small-scale experiments showed that acylation proceeded well in aqueous $NaHCO_3$ at room temperature, provided that more than 1 equiv of acylating agent was used to drive the reaction to completion. The requirement for excess *p*-nitrophenyl ester presumably reflected competition between acylation and hydrolysis. In the end we determined that 4 equiv each of $NaHCO_3$ and the acylating agent and a reaction time of 2–2.5 h were optimal. If haloacylation was allowed to continue beyond 2.5 h, if the temperature was raised to 50 °C, or if organic cosolvents such as MeOH or DMF were added, dark-colored side products were formed.

Several procedures for the purification of the *N*-haloacetyl derivatives were investigated. Precipitation from mixed organic solvents was successful with small amounts, but on a larger scale there was substantial formation of dark-green decomposition products. Similarly, decomposition occurred on attempted isolation of the haloacetyl compound as a sulfate or *p*-toluenesulfonate salt.[9] Unsuccessful attempts were also made to purify the haloacetamides by ion-exchange chromatography on DEAE-cellulose with NH_4HCO_3 as the eluent. In this case the considerable time that the product remained on the column at pH 8–9 led to extensive loss of alkylating activity, most probably because of hydrolysis to an *N*-glycolyl derivative. On TLC plates this apparent hydrolysis product was detected as a UV-absorbing spot which moved slightly behind the haloacetamide but gave a negative alkylation test. In the end the only way to purify the prouct with minimal decomposition was to pass it twice, in plain H_2O, through Bio-Gel P-2, a polyacrylamide size exclusion resin with a fractionation range of 100–1800 daltons. Removal of the dark-green impurity on Bio-Gel P-2 was very effective, as this material was eluted essentially in the first column volume. The rapid elution of this impurity suggests that it is a polymer formed via intermolecular alkylation of the 2,4-diaminopteridine moiety. The same explanation was given by Baker and co-workers[9] to account for the difficulties they experienced with *N*-haloacetyl derivatives of other 2,4-diaminopyrimidine systems.

While gel filtration was effective in separating the product from the dark-green polymer and from unchanged starting material, it did not entirely remove small amounts of salts, which in some instances coeluted with the product even after two passages through the column. It was possible to completely desalt the product by passing it through a third column, but this resulted in additional decomposition. For this reason we limited purification to two columns, at which point TLC showed a single UV-absorbing, strongly alkylating spot. Microchemical analysis showed the products were hydrated Na salts. There was also, in haloacetamides **2–4** a small excess of Na and either Br or Cl consistent with minor contamination (<10% by weight) of NaBr or NaCl, respectively. The effect of these small amounts of inorganic salt on biological experiments was expected to be negligible.

We had hoped initially to prepare not only the haloacetamides **2–5** but also those of the L-2,4-diaminobutanoic and L-2,3-diaminopropanoic acid analogues of MTX.[11,12] However, all efforts to obtain acceptable analyses for these shorter chained haloacetamides met with failure. Reactions with *p*-nitrophenyl bromoacetate or chloroacetate appeared to give the desired products according to TLC, but these products were much less stable than **2–5** and underwent extensive polymerization and/or hydrolysis during workup. Stability appeared to decrease in proportion to chain length and also seemed to vary according to whether the halogen was Br or Cl (the latter being more stable throughout the series).

The synthesis of compound **6** was accomplished by reaction of acivicin and 4-amino-4-deoxy-N^{10}-methylpteroic acid (MeAPA) in the presence of diethyl phosphorocyanidate.[13] We chose to use nonesterified acivicin because of concern that the chloroisoxazole might not survive acidic or alkaline ester cleavage conditions.[14,15] Two major products were formed, only one of which was mobile on cellulose TLC plates. The desired compound, **6**, was obtained in 25% yield by column chromatography on DEAE-cellulose with 3% NH_4HCO_3 as the eluent; the other product, whose identity was not established, remained bound.

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Table I. Physical Constants of N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl-N^{ω}-(haloacetyl)-α,ω-diaminoalkanecarboxylic Acids (Na Salts)

no.	n	X	% yield	mp, °C (dec)	calcd formula (analyses)
2	4	Br	32.5	265–275	$C_{23}H_{27}BrN_9O_4Na\cdot0.5NaBr\cdot6H_2O$ (C, H, N, Br, Na)
3	4	Cl	38.2	235–245	$C_{23}H_{27}ClN_9O_4Na\cdot0.08NaCl\cdot4H_2O$ (C, H, N, Cl, Na)
4	3	Br	34.7	230–235	$C_{22}H_{25}BrN_9O_4Na\cdot0.5NaBr\cdot4.8H_2O$ (C, H, N, Br)[b]
5	3	Cl	37.9	260–270	$C_{22}H_{25}ClN_9O_4Na\cdot3.5H_2O$ (C, H, N, Cl)[b]

[a] UV (H_2O): λ_{max} 302 nm (ϵ 23 154), 263 (17 706). [b] Na analyses were not obtained.

Enzyme Studies. The ability of the haloacetamides **2–5** to inhibit DHFR activity was assayed spectrophotometrically at 340 nm[16] with murine L1210/R81 enzyme[17] purified by MTX affinity chromatography.[18] From our previous measurements of time-dependent DHFR inactivation by 1,[2] it was assumed that inhibition in this rapid assay would reflect *noncovalent* binding. As shown in Table II, compounds **2–5** were all potent inhibitors, with IC_{50}s of <80 nM. The nonacylated precursors of **2–5** have IC_{50}s of 65 and 160 nM, respectively.[19] The N^{ϵ}-bromoacetyl derivative **2** had approximately the same IC_{50} as the parent amine, while the N^{ϵ}-chloroacetamide **3** was more potent. The N^{δ}-bromoacetyl and N^{δ}-chloroacetyl derivatives **4** and **5** were both stronger inhibitors than the nonacetylated L-ornithine analogue. The IC_{50}s of **3** and **5** were nearly the same as that of MTX itself, further confirming that structural modification of the "γ-terminal region" is well tolerated by the enzyme.[13,20]

Given the high chemical reactivity of the N^{ϵ}-haloacetyl groups in **2–5**, it seemed possible that longer incubation might result in *covalent* linkage as was observed earlier with **1**.[5] In order to determine the extent to which this occurs, we incubated purified DHFR with a 3- to 5-fold excess of the haloacetamide derivative, without NADPH, for periods of time ranging from 3 to 19 h. After treatment with 8 M urea to release noncovalently bound inhibitor, the enzyme was passed through a gel filtration column. An aliquot of the eluate (unmodified plus modified enzyme) was examined by UV. Increased absorbance at 300–330 nm in comparison with control enzyme was taken to signify covalently bound ligand. The UV spectrum of DHFR treated with the chloroacetamides **3** or **5** for 14 h was essentially the same as that of control enzyme, indicating that covalent modification had not occurred. In contrast, the spectrum of enzyme treated with the more reactive bromoacetamides **2** (19 h) or **4** (14 h) showed an increase in absorbance consistent with covalent binding. With the lysine derivative **2** this change was negligible at 3 h, confirming that covalent modification was time-dependent. From the pteridine extinction coefficient at 302 nm (Table I), the extent of covalent modification of DHFR was estimated to be 20 mol % with **2** and 60 mol % with **4**.

Since it was possible that NADPH might cause DHFR to assume a conformation more favorable for reaction with the haloacetamides, we also examined the UV spectrum of the enzyme after incubation with NADPH and excess **4** for 2.5 and 18 h. After trichloroacetic acid precipitation and treatment with 7 M guanidine hydrochloride at 70 °C, the enzyme was passed through a gel filtration column to remove any remaining traces of noncovalently bound drug. The gel-filtered protein was then analyzed for pteridine absorbance at 302 nm. As in the other experiment, absorbance at 302 nm increased with time. However, binding was no greater than when the incubation was conducted without NADPH. It thus appears that, at least for the L1210 enzyme, conformational changes induced by NADPH do not enhance alkylation by the ligand.

In order to gain additional evidence for covalent DHFR modification, we also treated aliquots of enzyme from the gel filtration columns (see above) with trichloroacetic acid, hydrolyzed the precipitated protein with HCl, and subjected the hydrolysate to quantitative amino acid analysis.[21] Unmodified enzyme served as the control, and results were compared with published sequencing data.[22,23] Even though the UV data were consistent with covalent modification, no change in amino acid composition could be demonstrated for enzyme treated with **2** or **4** in the absence of NADPH (binary complex) or for enzyme treated with **4** in the presence of NADPH (ternary complex). The apparent lack of reaction of the haloacetamides **2** and **4** with the lone Cys residue in the murine enzyme is of interest in view of our previous finding that **1** reacts with this amino acid in purified enzyme from chicken liver and human lymphoblasts.[5a,b] The failure of **4** and **5** to alkylate Cys in the L1210 enzyme may be due to the fact that these N^{δ}-(haloacetyl)ornithine derivatives contain one less CH_2 group than the N^{ϵ}-(haloacetyl)lysine analogue **1**. An alternative possibility is that the L1210 enzyme is unable to undergo the profound conformational change that would be needed to bring the Cys thiol group to within striking distance of the *N*-haloacetyl group. A plausible explanation for the lack of change in the apparent amino acid composition of the denatured enzyme despite the increase in UV absorption at 302 nm upon incubation with **2** or **4** may be that alkylation produced an acid-labile covalent modification. A possible site for such an alkylation might be the γ-carboxyl group of a glutamate residue (e.g., Glu-62 in the α C loop "insert" of the L1210 enzyme).[23] The resulting γ-carboxymethyl ester of glutamic acid would be expected to be unstable in hot HCl and would therefore not be detected during amino acid analysis. Carboxyl group alkylation at a glutamic acid residue (Glu-270) has been observed previously in the affinity labeling of bovine carboxypeptidase A_{γ}^{Leu} with *N*-(bromoacetyl)-*N*-methyl-L-phenylalanine.[24] Acid hydrolysis of the CNBr fragment containing the modified Glu-270 residue yielded L-glutamic acid and glycolic acid rather than γ-carboxymethyl L-glutamate.

Experiments were also performed with DHFR from *Candida albicans*[25] with the goal of determining how

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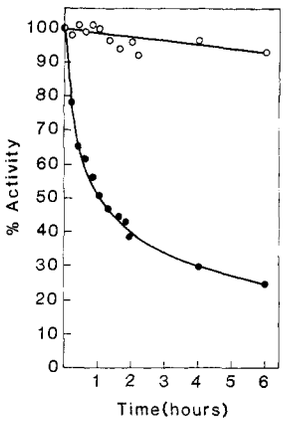


Figure 1. Time-dependent inactivation of DHFR from *Candida albicans* by N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-N^{δ}-(bromoacetyl)-L-ornithine (**4**) (binary complex, no NADPH). The enzyme was incubated in potassium phosphate, pH 7.4, in the presence of 100-fold excess drug (●) or without drug (○) for 6 h, passed rapidly through Sephadex G25 under nondenaturing conditions, and assayed for residual ability to reduce dihydrofolate with NADPH added (see Experimental Section).

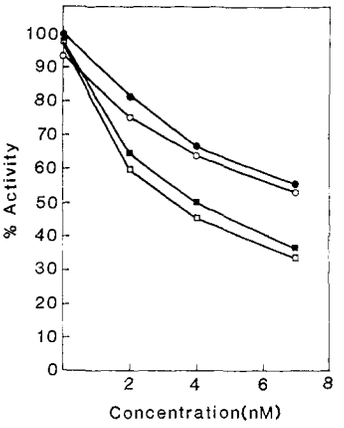


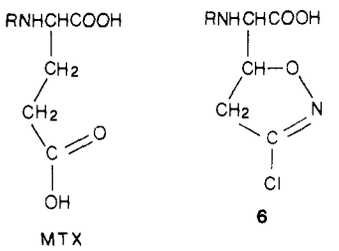
Figure 2. Titration of *Candida albicans* DHFR activity in the presence of MTX (■, □) or N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-(bromoacetyl)-L-lysine (**2**) (●, ○) and NADPH (ternary complex). The enzyme was incubated with 2, 4, or 7 nM inhibitor for 0 h (●, ■) or 2 h (○, □), and assayed for activity on addition of dihydrofolate (see Experimental Section).

compounds **2–5** interact with a fungal enzyme. In this case a nondenaturant procedure was used, and the extent of time-dependent inactivation was estimated by a functional assay of the enzyme's ability to catalyze dihydrofolate reduction. The enzyme was incubated with a 100-fold excess of inhibitor without NADPH, and at various times aliquots were passed rapidly through a gel filtration column. Control experiments showed that 99.9% of nonbound drug was retained while 75% of the enzyme activity was recovered. Residual activity after treatment with the inhibitor was measured spectrophotometrically by adding an aliquot of column eluate to a solution of dihydrofolate and NADPH. Irreversible DHFR inactivation was not observed with chloroacetamides **3** and **5**, but was observed with bromoacetamides **2** and **4** in qualitative agreement with the L1210 enzyme experiments. From a plot of residual enzyme activity vs. length of exposure to inhibitor, it was found that **4** produced 75% inactivation after 6 h (Figure 1). Similarly, inactivation of **2** was estimated to be 60% after 4 h (data not shown). Inhibition did not follow first-order kinetics, and 100% inactivation was not achieved. It should be noted that, while these results were consistent with at least partial covalent binding, amino acid analysis to identify the site of attack was not attempted.

Further studies were carried out to determine whether the haloacetamides would produce time-dependent inhibition of dihydrofolate reduction by the *Candida* enzyme in a functional assay (i.e., in the presence of NADPH). In this case the inhibitor was added in not more than 2-fold excess, and enzyme activity was measured spectrophotometrically after 2 h. As shown by the results with compound **2** (Figure 2), there was no difference between titration curves obtained at 0 and 2 h. In a control experiment using MTX instead of **2**, there was again no difference between 0 and 2 h. Similar results were obtained when **4** and MTX were compared (data not shown). It thus appeared that, under conditions of ternary complex formation, the haloacetamides probably did not bind covalently to the enzyme. Whether this reflects a greater protective effect by the NADPH for *Candida* enzyme than for murine enzyme, or is due to the fact that the inhibitor was used in insufficient excess in the *Candida* enzyme assay to compensate for chemical hydrolysis, is not known. The K_i for reversible inhibition of the *Candida* enzyme by compound **4** was determined from an analysis of steady-state reaction velocities.[26] The K_i of **4** was 0.32 nM, while that of MTX was 0.27 nM.

The ability of the MeAPA–acivicin conjugate **6** to inhibit DHFR under reversible conditions was assayed spectrophotometrically with enzyme from chicken liver and L1210 cells. The IC_{50} for inhibition of dihydrofolate reduction by the avian enzyme was 73 nM, whereas the IC_{50} for reduction by the murine enzyme was 46 nM. The IC_{50} of MTX was 50 and 55 nM, respectively. Thus, replacement of the glutamate moiety in MTX by acivicin did not significantly alter reversible binding. DHFR from both chicken liver and L1210 cells contains a thiol group, which might be expected to react with the chloroisoxazole moiety. However, because **6** was only modestly active against L1210 cells in culture (see below), we did not attempt to determine whether it can produce time-dependent irreversible inactivation of DHFR.

As indicated in the partial structures below, the side chains in MTX and **6** can be aligned in a manner that allows the OH, C=O, and γ-CH_2 groups of the MTX and the Cl, C=N, and γ-CH_2 groups of **6** to occupy approximately the same space. Moreover the Cl—C=N system corresponds to the "γ-terminal" region of MTX, which has been shown to be relatively tolerant to structural change for DHFR binding. On the other hand, the amino acid moiety in **6** is β-substituted, and it was unknown prior to this work how a polar atom near the critically important α-carboxyl might affect binding. Our results suggest that β-substitution on the side chain of MTX also may be tolerated, though more extensive structure–activity analysis is needed to define the limits of this tolerance.



Cell Growth Studies. Cytotoxicities of compounds **2–5** were determined against wild-type L1210 cells and against the MTX-resistant line L1210/R81, which has been shown

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Table II. Dihydrofolate Reductase Inhibition and Cell Growth Inhibition by N^{ϵ}-(Haloacetyl)-L-lysine and N^{δ}-(Haloacetyl)-L-ornithine Analogues of MTX

compd	n	X	DHFR (L1210/R81)[a] IC_{50}, nM	cell growth:[b,c] IC_{50}, μM L1210	L1210/R81
2	4	Br	72	0.096	240 (2500)
3	4	Cl	32	0.033	93 (2800)
4	3	Br	46	0.126	155 (1200)
5	3	Cl	32	0.062	105 (1700)
MTX			25	0.005	200 (44000)

[a] See reference 16 for details of the assay method. The following reactants were used in the assay: 49 nM DHFR, 50 μM dihydrofolate, and 75 μM NADPH in 50 mM Tris chloride, pH 7.5. Preincubation of the DHFR with inhibitor and NADPH was for 2–3 min prior to addition of dihydrofolate. Changes in absorbance were read at 340 nm. [b] See reference 17 for cell culture conditions and the properties of the L1210/R81 cell line. [c] Values in parentheses represent fold resistance and were calculated by dividing the IC_{50} (L1210/R81) by the IC_{50} (L1210).

to have a nearly total absence of MTX active transport in addition to a moderate (35-fold) increase in content of DHFR with normal affinity for MTX.[17] The cells were incubated in RPMI medium at 37 °C for 48 h. The medium contained no 2-mercaptoethanol, which would have rapidly destroyed alkylating activity. As indicated in Table II, the haloacetamides had IC_{50}s ranging from 33 nM (**4**) to 126 nM (**3**). The nonacylated L-lysine and L-ornithine analogues were previously found to have IC_{50}s of 400 and 1300 nM, respectively.[12] Thus, N-haloacetylation produced a very substantial increase in molar potency in the cytotoxicity assay. This was certainly not due only to reversible binding to DHFR, since the parent compounds and their haloacetamides showed comparable activities against purified enzyme in the short-term spectrophotometric assay. It is possible that removal of the positive charge from the side chain terminal via acylation increased uptake across the cell membrane, and that this contributed to the greater activity of the haloacetamides. Two other possibilities that cannot be ruled out at present are that there may be at least some irreversible binding to DHFR, and that there may be covalent binding to membrane-associated proteins that are involved in the active transport of reduced folates.[27] Such binding could result in diminished ability of the cell to take up reduced folates from the culture medium, thereby potentiating the anti-DHFR effect. The latter possibility is attractive in view of the fact that the haloacetamides are likely to encounter cell-surface proteins before reaching the cytoplasm. Henderson and co-workers[28] have explored this potential therapeutic modality in their work on *N*-hydroxysuccinimide esters of MTX, which are thought to react covalently with nucleophilic residues on the MTX/reduced folate carrier. While MTX *N*-hydroxysuccinimide esters would be expected to react most readily with basic lysine residues on the carrier, haloacetamides might be more likely to attack thiol groups, for which a vital role in the active transport of MTX and reduced folates has been demonstrated in studies with SH-reactive probes.[29]

When compounds **2–5** were tested as inhibitors of the growth of L1210/R81 cells (Table II), IC_{50}s were found to range from 93 μM (**3**) to 240 μM (**2**). There was for the most part a good correlation betwen the IC_{50}s for L1210/R81 cell growth inhibition and those for L1210/R81 DHFR inhibition, and also between the IC_{50}s for growth inhibition of wild-type vs. resistant cells. The L1210/R81 cells showed 1000- to 3000-fold resistance to the haloacetamides, where the resistance of these cells to MTX was >40 000-fold. Furthermore, three of the haloacetamides (**3–5**) proved more potent than MTX against the resistant line. It thus appears that, whatever the details of their action, these compounds can partly overcome MTX resistance in these cells.

The ability of the acivicin analogue **6** to inhibit L1210 cell growth in culture was compared to those of the haloacetamides **2–5** and of MTX. The IC_{50} of **6** was found to be 0.38 μM. Introduction of an acivicin moiety therefore led to approximately the same loss of activity in cell culture, relative to MTX, as did the introduction of an N^{ϵ}-(haloacetyl)-L-lysine or N^{δ}-(haloacetyl)-L-ornithine side chain. Thus, even though there was only a slight difference in the IC_{50} for DHFR inhibition between **6** and MTX, there was a substantial difference in growth inhibitory potency consistent with poor uptake. It is also possible, however, that the cloroisoxazole did not remain intact, and that **6** was partly converted, either in the cell or extracellularly, to products with low DHFR affinity. The fact that **6** differs from MTX in not being able to form γ-polyglutamates might also be expected to decrease activity. However we have shown that other MTX analogues that cannot form γ-polyglutamates are not necessarily less toxic than MTX to cells exposed continuously to drug.[30] Thus the inability of **6** to undergo polyglutamylation is probably less important in a culure assay than the ease with the compound can enter the cell.

While the ability of haloacetamides **2–5** and of the acivicin analogue **6** to inhibit DHFR activity and cell growth is interesting, progress beyond the present level of experimentation is likely to be hampered by the difficulty of preparation and purification of these compounds, by the possibility that IC_{50}s determined in culture may reflect in part the action of dehalogenated derivatives formed by hydrolysis in the culture medium, and because only a fraction of the drug may survive long enough after injection into a whole animal to reach the tumor site. The concept of active-site-directed DHFR inhibition by analogues of the classical antifolate type nonetheless remains theoretically attractive, provided that solutions to these complex problems can be found.

Experimental Section

Melting points were determined in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, MA) or a Fisher-Johns hot-stage apparatus (Fisher Scientific, Boston, MA) and are uncorrected. IR spectra were obtained on a Perkin-Elmer Model 781 double-beam spectrophotometer, and UV spectra were measured on a Varian Model 210 UV/visible instrument. Thin-layer chromatography (TLC) was on Whatman MK6F and Baker 250F silica gel plates containing a fluorescent indicator, and spots were visualized under 254-nm illumination. The plates were developed in two solvent systems (A, $CHCl_3$–MeOH–AcOH, 15:5:1; B, *n*-BuOH–AcOH–H_2O, 3:1:1). Microanalyses were performed by Galbraith Laboratories, Knoxville, TN, or Multichem Laboratories, Lowell, MA, and were within ±0.4% of the theoretical values unless otherwise noted. reagent grade solvents were redistilled and stored over Davison 4A molecular sieves (Fisher Scientific, Boston, MA). Bio-Gel P-2 was purchased from Bio-Rad Laboratories, Richmond, CA. Metho-

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trexate analogues containing a basic amino acid side chain were made according to procedures recently described.[12] *p*-Nitrophenyl chloroacetate and *p*-nitrophenyl bromoacetate were synthesized according to Samant and Sweet.[31] Acivicin was a generous gift from the Upjohn Co., Kalamazoo, MI. MeAPA was prepared as previously described,[12] and diethyl phosphorocyanidate was purchased from Aldrich, Milwaukee, WI. The DMF used in coupling reactions was dried over Davison 4A molecular sieves (Fisher, Boston, MA). Biochemicals were from Sigma, St. Louis, MO.

Synthesis of N^{α}-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-N^{ω}-(haloacetyl)-α,ω-diaminoalkanecarboxylic Acids (Na Salts). General Procedure. The starting amino acid (0.3 mmol) was added to a stirred solution of $NaHCO_3$ (101 mg, 1.2 mmol) in H_2O (3 mL), and to the solution was added the appropriate *p*-nitrophenyl haloacetate ester (1.2 mmol). The mixture was stirred at room temperature and the progress of the reaction was followed by TLC. When the starting material was almost completely consumed (ca. 2 h), *p*-nitrophenol and excess acylating agent were removed by extraction with EtOAc (4 × 20 mL) and then with Et_2O (15 mL). Traces of organic solvents were removed from the aqueous phase by rotary evaporation at room temperature, the solution was applied onto a Bio-Gel P-2 column (100 × 2.5 cm) packed in H_2O, and products were eluted with H_2O with protection from light. A greenish fraction assumed to contain polymeric quaternized material eluted first and was followed by a major yellow band containing mainly one product according to TLC. A slower moving yellow band contained a small amount of unchanged starting amino acid. The product-containing fraction was lyophilized and passed through a second Bio-Gel P-2 column to obtain the final TLC-homogeneous product in final yields of 30–40% (Table I). The haloacetamide derivative was stored in a tightly capped vial under N_2 at −70 °C in the presence of a dessicant and was stable under these conditions for many months.

α-[N-(4-Amino-4-deoxy-N^{10}-methylpteroyl)amino]-3-chloro-4,5-dihydro-5-isoxazoleacetic Acid (6, MeAPA–Acivicin). MeAPA (432 mg, 1.2 mmol) was added, in small portions as it dissolved, to a solution of diethyl phosphorocyanidate (588 mg, 3.6 mmol) and Et_3N (364 mg, 3.6 mmol) in dry DMF (40 mL). The mixture was stirred overnight and to it were added acivicin (190 mg, 1.1 mmol) and Et_3N (505 mg, 5.0 mmol). A homogeneous solution was obtained after 6 h. Stirring was continued for 3 days, and after rotary evaporation the residue was taken up in H_2O with enough NH_4OH added to bring the final pH to 9. A small amount of undissolved material was removed by filtration through coarse filter paper, and the filtrate was chilled and treated with 10% AcOH until a solid formed. The solid was collected and dried with the aid of a lyophilizer; crude yield 538 mg. TLC showed two products, R_f <0.1 and R_f 0.5. This crude material was mixed with 3% NH_4HCO_3 and DEAE-cellulose (ca. 8 g) which had previously been equilibrated with the same buffer. The mixture was stirred a few minutes and filtered, and the filter cake was washed liberally with 6% NH_4HCO_3 until the yellow color of the wash solution began to fade. TLC at this point showed a single major spot at R_f 0.5 and no spot at the origin. The 6% NH_4HCO_3 wash solution was freeze-dried and the residue was redissolved and applied onto a column of DEAE-cellulose (25 g, packed in 3% NH_4HCO_3 and washed to neutrality with H_2O). The column was eluted first with H_2O and then successively with 1.5% and 3% NH_4HCO_3. Pooled fractions containing **6** were freeze-dried; yield 240 mg (25%); IR (KBr) ν 3450, 1640, 1610 (sh) cm^{-1}; UV (pH 7.4) λ_{max} 259 nm (ϵ 24 700), 301 (25 000), 373 (8990); UV (0.1 N HCl) λ_{max} 239 nm (infl, ϵ 20 000), 307 (24 800). Anal. ($C_{20}H_{20}ClN_9O_4 \cdot H_2O$) C, H, N (Cl: calcd, 6.77; found, 7.19).

Reversible Enzyme Inhibition Assay. The ability of compounds to inhibit dihydrofolate reduction by purified DHFR from L1210/R81 cells[17] was measured spectrophotometrically at 340 nm as described earlier.[16] The enzyme was purified to homogeneity by chromatography on a MTX–agarose affinity column.[18] The following reactants were used: 50 nM DHFR, 50 μM dihydrofolate, and 75 μM NADPH in 50 nM Tris chloride, pH 7.5. The enzyme was preincubated with inhibitor and NADPH for 2–3 min prior to addition of dihydrofolate.

Irreversible Binding to DHFR from L1210 Cells. A. Binary Complex. An excess of test compound, dissolved in a minimal volume of Me_2SO, was added to purified enzyme in 1 mL of 0.1 M potassium phosphate buffer, pH 7.5, containing 10% glycerol. The final Me_2SO concentration in the mixture was 0.25%. In the assay of **2**, a 3-fold molar excess of compound was added to 794 μg (37 nmol) of enzyme; in the assay of **3–5**, a 5-fold molar excess of compound was added to 365 μg (17 nmol) of enzyme. Aliquots were removed from the assay mixtures at various times (**2**, 3 and 19 h; **3–5**, 14 h), and urea was added to a final concentration of 8 M. After 10 min of incubation at room temperature, each solution was passed through a Bio-Gel P-6DG column (1.5 × 6 cm) preequilibrated with 8 M urea in 50 mM Tris chloride, pH 7.5. Protein-containing eluates were pooled and treated with 10% trichloroacetic acid, and the protein precipitate was washed with H_2O and acetone to remove salts and urea and then resuspended in 30% AcOH. A 10% aliquot was set aside for amino acid analysis, and a UV-absorption spectrum was obtained on the rest. Unmodified enzyme subjected to identical treatment was used as the control. The amount of pteridine ligand per mole of enzyme was estimated in each case from the absorbance intensity at 302 nm and an approximate molar extinction of 22 100 for the inhibitor at this wavelength.

Ternary Complex. A second experiment was performed to examine the effect of NADPH on the interaction of compound **4** with L1210 enzyme. A 4-fold excess of **4** was added to 7 nM of enzyme in 0.4 mL of 50 mM triethanolamine, pH 7.0, containing 350 nM of NADPH. An identical mixture was prepared without NADPH. After 2.5 and 18 h, an aliquot containing 3.5 nM of enzyme was removed from each mixture, and trichloroacetic acid was added. After centrifugation, the pellet was washed with H_2O, and incubated at 70 °C for 10 min with 7 M guanidine hydrochloride in 50 mM triethanolamine (0.25 mL). The denatured enzyme was passed through a Bio-Gel P-6 column (1.5 × 9 cm) in the same buffer in order to remove any residual nonbound drug, and protein-containing fractions were precipitated with trichloroacetic acid, washed with H_2O to remove salts, and redissolved in 30% AcOH. A 20% aliquot was set aside for amino acid analysis, and the rest was used to record the UV spectrum. The amount of bound drug per enzyme molecule was estimated spectrophotometrically.

Quantitative amino acid analyses were carried out with each batch of drug-treated enzyme as previously described,[21] and results were normalized for 18 leucines. Data obtained with untreated control enzyme were in agreement with published values,[23] and values obtained with drug-treated enzyme did not differ from controls. *S*-(Carboxymethyl)-L-cysteine was not detected in hydrolysates of the treated enzyme, as would be required if the haloacetamides had alkylated a Cys residue.

Irreversible Binding to DHFR from *Candida albicans*. A. Binary Complex. A 100-fold molar excess of the inhibitor was added to a solution containing 30 nM enzyme in 0.1 M potassium phosphate buffer, pH 7.4. The mixture was incubated at 30 °C in the dark, and 0.2-mL aliquots were removed at regular intervals and immediately subjected to centrifugal filtration through a Sephadex G25 minicolumn in a 1-mL disposable syringe (0.9-mL packed gel volume, prewashed with 0.2 mL of 1% bovine serum albumin by centrifugation). The eluted enzyme was then assayed spectrophotometrically for activity at 30 °C. The assay mixture contained 60 μM NADPH, 45 μM dihydrofolate, 12 mM 2-mercaptoethanol, and 0.1 M imidazole hydrochloride, pH 6.4, in a final volume of 1 mL. Activity was plotted as function of time and compared with the results with sham-incubated control enzyme. Time-dependent loss of activity was expressed as a percent of control. Enzyme recovery from the Sephadex G25 column was found to be 75%, and enzyme activity over the period of the experiment remained unchanged in the absence of inhibitor. When a sample of [^{3}H]MTX was applied to the column without enzyme, 99.9% of it remained bound.

B. Ternary Complex. Solutions containing 4 nM enzyme and 64 μM NADPH in 0.1 M potassium phosphate buffer, pH 7.4, were prepared, and the test compound was added to a final concentration of 2, 4, or 7 nM. Control solutions contained either no inhibitor or the same concentrations of MTX. After 0 and 2 h, 980-μL aliquots were removed and assayed spectrophoto-

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metrically at 340 nm for activity. The assay was performed at 30 °C in a mixture containing 64 μM NADPH, 45 μM dihydrofolate, 12 mM 2-mercaptoethanol, and 0.1 M potassium phosphate, pH 7.4, in a final volume of 1 mL. The reaction was initiated by addition of the dihydrofolate in a volume of 20 μL. Apparent K_is were obtained from Lineweaver–Burk double-reciprocal plots of the data for reversible inhibition (i.e., at time zero).

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Synthesis and β-Lactamase Inhibitory Properties of 2β-[(1,2,3-Triazol-1-yl)methyl]-2α-methylpenam-3α-carboxylic Acid 1,1-Dioxide and Related Triazolyl Derivatives[1]

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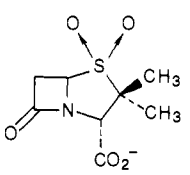
Benzhydryl 2β-[(1,2,3-triazol-1-yl)methyl]-2α-methylpenam-3α-carboxylate 1,1-dioxide was prepared by heating benzhydryl 2β-(azidomethyl)-2α-methylpenam-3α-carboxylate 1,1-dioxide with (trimethylsilyl)acetylene. The ester group was removed by hydrogenolysis to give sodium 2β-[(1,2,3-triazol-1-yl)methyl]-2α-methylpenam-3α-carboxylate 1,1-dioxide (**3i**, YTR-830), which was found to be a potent inhibitor of various bacterial β-lactamases. A series of related compounds was prepared in a similar way, and all of these compounds show excellent β-lactamase inhibitory properties.

The production of β-lactamase is a common defence mechanism of bacteria to β-lactam antibiotics. The enzyme (β-lactamase) catalyzes the hydrolysis of β-lactam antibiotics to the inactive penicilloic acids, and bacteria possessing this enzyme are therefore resistant to the killing effects of clinically important β-lactam antibiotics. Recently, a new approach effective against this defence mechanism of bacteria has been developed by utilizing "suicide" or "mechanism-based" irreversible inactivation of the enzyme. The significant breakthrough in this area came only in 1976 with the discovery of clavulanic acid,[2] which proved to be a very effective synergist. Over the past 8 years a number of naturally occurring and semisynthetic β-lactam compounds that inhibit or inactivate β-lactamase have been reported in the literature.[3] Penicillanic acid sulfone[4] **1** (sulbactam), first reported by the Pfizer group, is a potent synergist when used together with β-lactam antibiotics against resistant bacterial strains, thereby protecting the antibiotic from hydrolytic destruction by the enzyme.



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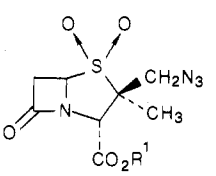

2

From investigations of the interaction of the enzyme with a number of β-lactamase inactivators including clavulanic acid, penam sulfones, and 6-halopenams, a possible mechanism of suicide inactivation has been proposed[3a] and is outlined in Scheme I.

The catalytic activity of the enzyme can be disrupted if the initially formed acyl–enzyme complex [B] can react by path a or path b rather than the normal deacylation pathway as illustrated in Scheme I. In a number of instances a branched reaction pathway (a or b) has been favored, and there is much evidence supporting the notion that the formation of a stable β-aminoacrylate system [F] is often responsible for enzyme inactivation.

In recent years since the β-lactamase has become an important target in β-lactam antibiotic research, the search for more effective inactivators of this enzyme has escalated. A recent report[5] from our laboratory has described the synthesis of 2β-(azidomethyl)-2α-methylpenam-3α-carboxylate 1,1-dioxide (**2**), which also possesses β-lactamase inhibitory property;[6] $IC_{50} = 4.0 \times 10^{-6}$ M. Since alkyl azides are remarkably reactive toward various reagents,[7,8] we have explored the feasibility of transforming the azido group of **2** into novel triazole systems **3** (Scheme II). These compounds, **3**, have proven to be even more effective β-lactamase inhibitors.

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