Table I—Effects of Test Compounds on Ehrlich Ascites Carcinoma[a] Growth

Compound	Dose, mg/kg/day ip	Survival at Day 9	Ascrit[b]	Ascites Volume, Mouse	Inhibition[c], %
0.05% Polysorbate 80		34/40	33.6 ± 8.7	1.8 ± 1.02	0.0
V[d]	5	8/8	22.0	2.45	57.1
	10	8/8	0.0	0.0	100.0
	20	8/8	35.7	0.88	81.8
III	10	8/8	43.8	0.15	80.0
IV	10	6/6	21.2	2.2	0.0
6-Mercaptopurine[e]	200	6/6	0.3	0.7	99.6

[a] 2×10^6 cells were injected intraperitoneally into 6 or 8 male CF_1 mice on day 0. The drug was administered from day 1 to 8. On day 9 the mice were sacrificed and the experiment was evaluated. [b] Packed cell volume as a percent. [c] Greater than 80% inhibition is required for significant activity. [d] Compound V is 3-chloromethylthiochromone-1,1-dioxide (6). [e] Sigma Chemical Co.

10 mg/kg/day in mice, *versus* Ehrlich ascites carcinoma tumor growth, was found to be only marginally active (80% inhibition of tumor growth) relative to many other compounds reported previously (6) (Table I). Several of these compounds, such as 3-chloromethylthiochromone-1,1-dioxide (V) (highly active in the test, giving 100% inhibition), could conceivably give rise to compound III *in vivo* by bioreduction and hydrogen chloride elimination (1, 3). The low activity observed with compound III may result from *in vivo* dimerization or other instability such as participation in Michael type reactions. The dimer (IV) was completely inactive in the antitumor test (Table I).

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Diazoketone and Chloromethylketone Analogs of Methotrexate as Potential Antitumor Agents

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Abstract □ The synthesis of 4-amino-4-deoxy-N^{10}-methylpteroyl-(6-diazo-5-oxo)-L-norleucine and 4-amino-4-deoxy-N^{10}-methylpteroyl-(6-chloro-5-oxo)-L-norleucine, analogs of methotrexate in which the γ-carboxyl group is replaced by a diazoketone and a chloromethylketone, respectively, was carried out. The analogs inhibited the growth of leukemia L-1210 cells in culture by 50% at 4×10^{-7} *M* and 2×10^{-7} *M*, respectively, and were effective inhibitors of the synthesis of thymidylate in L-1210 cells *in vitro* ($I_{50} = 3 \times 10^{-6}$ *M*), exhibiting significant antifolate activity. The results demonstrated the feasibility of introducing chemically reactive groups at the γ-position of pteroyl glutamates with retention of biological activity. However, in the systems investigated thus far, there was no evidence of covalent bond formation due to these reactive groups at the active sites of the enzymes.

Keyphrases □ Methotrexate—diazoketone and chloromethylketone as potential antitumor agents □ Antitumor agents—potential, diazoketone and chloromethylketone analogs of methotrexate □ Analogs—diazoketone and chloromethylketone, of methotrexate, potential antitumor agents

Many structural analogs of the clinically useful antitumor agent methotrexate (4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid, amethopterin) (1), modified at the carboxyl groups of its glutamic acid moiety, have been prepared in the past in attempts to alter the membrane transport properties and to improve the tissue distribution and selectivity of the drug, as well as to circumvent drug resistance. This class of methotrexate derivatives includes a variety of α- and γ-monoesters (2), diesters (3), amides (4), and peptides (4, 5) and also analogs in which the carboxyl groups are replaced by hydrogen (6–8), hydroxymethyl, or methyl (9) groups. As was observed for methotrexate analogs modified at other parts of the molecule (8, 10), with the possible exception of 10-deazaaminopterin

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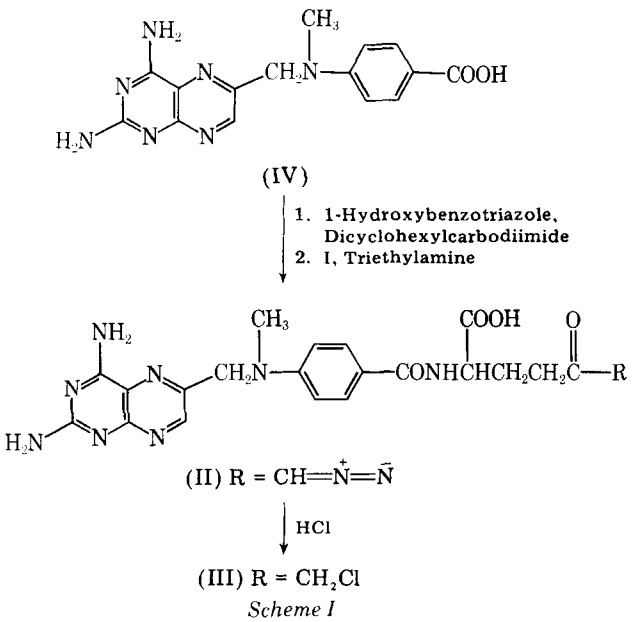
(10), none of these derivatives proved more effective than methotrexate. Modification at the α-position generally led to a marked decrease in biological activity including binding to the primary target dihydrofolate reductase, whereas substitution at the γ-carboxyl was better tolerated (4–10), with enzyme inhibitory activities approaching that of the parent drug.

The feasibility of introducing chemically reactive groups capable of covalent bond formation into the more permissive γ-position of pteroyl glutamates was explored. This type of modification may lead to the development of new active-site directed inhibitors (11) of folate metabolizing enzymes with potential antitumor activity. The replacements of the γ-carboxyl of methotrexate by a diazoketone and a chloromethylketone were chosen as prototypes of this modification. The diazoketone analog of glutamic acid, the antitumor antibiotic 6-diazo-5-oxo-L-norleucine (I) is a potent, irreversible inhibitor of glutamine amidotransferases (12). α-Haloketone analogs of various substrates are widely used for the affinity labeling of enzyme active sites (11–13).

In this report the synthesis and *in vitro* biological activities of the methotrexate analogs 4-amino-4-deoxy-N^{10}-methylpteroyl-(6-diazo-5-oxo)-L-norleucine (II) and 4-amino -4- deoxy-N^{10}-methylpteroyl-(6-chloro-5-oxo)-L-norleucine (III) are described. The results demonstrate that methotrexate can be substituted at the γ-position of its glutamate moiety with diazoketone and chloromethylketone functionalities, and that these modifications permit effective cellular uptake and expression of antifolate activity.

RESULTS AND DISCUSSION

Chemistry—The synthesis of the target compound (II) was accomplished by coupling 4-amino-4-deoxy-N^{10}-methylpteroic acid (14) (IV) with I using dicyclohexylcarbodiimide and 1-hydroxybenzotriazole *via* a modification of the peptide coupling procedure of Konig and Geiger (15, 16) (Scheme I). This coupling procedure, requiring milder conditions, was selected over the mixed anhydride method generally adopted for the coupling of IV with a variety of amino acid esters (6), mainly because of the known instability of I (17) to heat and pH extremes. Compound II displayed strong IR absorption bands at 2106 cm^{-1}, characteristic of the diazo moiety, and at 1633 and 1605 cm^{-1}, indicative of the ketone and amide, respectively. The NMR spectrum was consistent with the assigned structure; it included a singlet at 5.99 ppm associated with the methine proton of the diazoketone and a multiplet, strongly resembling a doublet, at 7.98 ppm assigned to the NH proton of the amide group.



Scheme I

Treatment of II with dry hydrogen chloride afforded the hydrochloride salt of the chloromethylketone analog (III), illustrated in Scheme I. The IR spectrum of III included a strong band at 1725 cm^{-1}, characteristic of chloromethyl ketones, and at 1610 cm^{-1}, assigned to the amide. The NMR spectrum included a singlet at 4.48 ppm, assigned to the methylene protons of the chloromethylketone, in accordance with the assigned structure.

Biological Results—The growth of leukemia L-1210 cells in culture (18) was inhibited 50% by II and III at 4×10^{-7} *M* and 2×10^{-7} *M*, respectively. In L-1210 cells *in vitro*, both compounds showed the same antifolate activity as measured by the *in situ* thymidylate synthetase assay (19) ($I_{50} = 3 \times 10^{-6}$ *M*). The potency of II and III was within an order of magnitude of that of methotrexate. The activity in both assay systems may be attributed to methotrexate-like activity involving inhibition of intracellular dihydrofolate reductase.

In preliminary studies of the effects of II and III in isolated enzyme systems, the thymidylate synthetases of *Lactobacillus casei* (20) and human blast cells (21), dihydrofolate reductase of *L. casei* (22), and folylpolyglutamate synthetase of rat liver (23), no irreversible inactivation was demonstrated. In these systems methotrexate exerted reversible inhibitory activity, with the exception of folylpolyglutamate synthetase, for which methotrexate is a substrate (23). Using various experimental tumor and isolated enzyme systems in comparison with methotrexate, further studies of II and III are in progress.

The synthetic procedures described in this paper are currently being applied with appropriate modifications to the preparations of diazoketone and chloromethylketone analogs of folic acid and its derivatives.

EXPERIMENTAL[1]

4-Amino-4-deoxy-N^{10}-methylpteroyl- (6-diazo-5-oxo) -L-norleucine (II)—To a solution of 4-amino-4-deoxy-N^{10}-methylpteroic acid (IV) (14), (180 mg, 0.5 mmole) in 10 ml of dimethyl sulfoxide–tetrahydrofuran (1:1) cooled to 0° was added 1-hydroxybenzotriazole (67.2 mg, 0.5 mmole) and dicyclohexylcarbodiimide (102.5 mg, 0.5 mmole). The reaction mixture was stirred for 1 hr at 0° and 1 hr at room temperature. To this mixture, kept in the dark, was added a solution of 6-diazo-5-oxo-L-norleucine (86.2 mg, 0.5 mmole) in dimethyl sulfoxide (2 ml) and water (1.5 ml). The reaction coupling time was 3 hr, during which the pH of the reaction mixture was monitored and maintained at 7.9–8.0 by the addition of the required amount of a dilute solution of triethylamine–dimethyl sulfoxide–tetrahydrofuran (2:1:5). After 3 hr, the reaction mixture was cooled to −15° overnight and filtered. Tetrahydrofuran was removed from the filtrate by evaporation which was followed by filtration and evaporation of the filtrate *in vacuo* to dryness. The residue was stirred with *N,N*-dimethylformamide and filtered to afford a clear solution which was concentrated to half of its volume, cooled overnight (−15°), and filtered. The filtrate was evaporated to dryness and triturated with ethyl acetate; subsequent filtration followed by washing with tetrahydrofuran, water, and acetone afforded a yellow solid (II). The compound was homogeneous by TLC (silica gel, dimethylsulfoxide–H_2O, 1:1), yield 53% (71% based on unrecovered IV). IR (KBr): 2106 ($N^+{=}N^-$), 1633 ($COCHN_2$), 1605 (CONH) cm^{-1}; NMR (dimethyl sulfoxide-d_6): δ 3.22 (s, 3H, N—CH_3), 4.32 (m, 1H, α—CH), 4.79 (s, 2H, CH_2—N), 5.99 (s, 1H, $CH{=}N^+{=}N^-$), 6.67 (s, 2H, NH_2), 6.83 and 7.72 (2d, 4H, C_6H_4), 7.50 (broad s, 2H, NH_2), 7.98 (m, resembling a doublet, 1H, CONH), and 8.58 (s, 1H, C_7H) ppm.

Anal—Calc. for $C_{21}H_{22}N_{10} \cdot 0.5\ H_2O$: C, 51.74; H, 4.72; N, 28.75. Found: C, 52.04; H, 4.91; N, 27.71. (The low value for nitrogen is not unusual in the elemental analysis of diazo compounds.)

4-Amino-4-deoxy-N^{10}-methylpteroyl- (6-chloro-5-oxo) -L-norleucine hydrochloride (III). Compound II (115 mg, 0.24 mmole) was dissolved in dry *N,N*-dimethylformamide (6.5 ml) and cooled in an ice-water bath. Dry hydrogen chloride was bubbled into the cooled solution for 10 min. The deep brown solution was stirred for 1 hr at 4° and then poured into cold anhydrous diethyl ether (35 ml). The crude product was purified by repeated triturations with cold (4°) anhydrous diethyl ether, and finally with ether–ethanol mixture (10:1), to give a brown oil. Con-

[1] IR spectra were obtained using a Nicolet Model 7000 Fourier transform IR spectrometer on samples prepared in a potassium bromide pellet. NMR spectra were obtained using a Varian FT-80 instrument. Compounds were dissolved in dimethyl sulfoxide-d_6 from commercial sources with tetramethylsilane as the internal standard.

tinued treatment with several portions of cold anhydrous ether, followed by filtration in a dry box under nitrogen, gave III as a pale yellow solid, yield 98%. IR (KBr): 1725 ($COCH_2Cl$), 1610 (CONH) cm^{-1}; NMR (dimethyl sulfoxide-d_6): δ 3.26 (s, 3H, N—CH_3), 4.48 (s, 2H, CH_2Cl), 4.88 (broad s, 3H, CH_2 and α—CH), 6.83 and 7.75 (two d, 4H, C_6H_4), 7.95 (s, 2H, CONH), and 8.75 (s, 1H, C_7H) ppm. An analytical sample was obtained by drying over phosphorus pentoxide *in vacuo*.

Anal.—Calc. for $C_{21}H_{23}ClN_8O_4 \cdot HCl \cdot 4H_2O$: C, 42.36; H, 5.42; N, 18.82; Cl, 11.91. Found: C, 42.61; H, 5.47; N, 19.08; Cl, 11.73.

Cell Culture Studies—Growth inhibition of murine leukemia L-1210 cells in culture was determined as described previously (18). The cells were maintained in a medium[2] supplemented with 10% calf serum[3] and antibiotics (penicillin and streptomycin). The cultures were incubated at 37° for 40 hr. During this time, the density in the control cultures increased from 1.5×10^5 to $1.2–1.5 \times 10^6$ cell/ml. Cell viability was determined by the trypan blue dye exclusion technique.

Cellular Thymidylate Synthetase Assay—Studies of the inhibition of intracellular thymidylate synthesis were carried out using murine leukemia L-1210 cells suspended in modified Eagle's media (without folate). The L-1210 cell line was maintained by weekly passage of 10^5 cells into DBA/2HA mice and harvested 6 days after inoculation.

The cellular radioisotopic assay of thymidylate synthetase activity was performed as described previously (19) by measuring the extent of tritium released into water from [5-^{3}H]deoxyuridine[4] (specific activity 22 Ci/mmole). Radioactivity was determined by liquid scintillation counting techniques[5] using a counter and scintillation cocktail[6] with 30–35% counting efficiency.

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[2] RPMI 1640 medium.
[3] Dialyzed, GIBCO No. 644.
[4] Moravek Biochemicals.
[5] Packard Model 3255 TriCarb Counter.
[6] Fisher, Scinti Verse.

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