Synthesis and Antifolate Properties of 10-Alkyl-5,10-dideaza Analogues of Methotrexate and Tetrahydrofolic Acid

Joseph I. DeGraw,*,† Pamela H. Christie,† Roy L. Kisliuk,‡ Yvette Gaumont,‡ and Francis M. Sirotnak§

Bio-Organic Chemistry Laboratory, SRI International, Menlo Park, California 94025, Department of Biochemistry, Tufts University Health Sciences Campus, Boston, Massachusetts 02111, and Memorial Sloan Kettering Cancer Center, New York, New York 10021. *Received December 1, 1988*

Synthesis of the 10-methyl and 10-ethyl analogues of 5,10-dideazatetrahydrofolic acid (DDTHF), a potent inhibitor of glycinamide ribotide (GAR) formyltransferase, is reported. Key intermediates in the process were 10-methyl- and 10-ethyl-4-amino-4-deoxy-5,10-dideazapteroic acid. Condensation of the piperidine enamines of branched 4-(*p*-carbomethoxyphenyl)butyraldehydes with (acetoxymethylene)malononitrile afforded 1,1-dicyano-4-piperidinobutadiene **5a,b**. Subsequent reaction with alcoholic ammonium hydroxide yielded the appropriately substituted 2-amino-3-cyanopyridines **6a,b**. Ring closure with guanidine gave 10-methyl- and 10-ethyl-4-amino-4-deoxy-5,10-dideazapteroic acids (**7a,b**). Coupling with diethyl glutamate followed by ester hydrolysis afforded 10-alkyl-5,10-dideazaminopterin analogues **9a,b**. Hydrolysis of the 4-amino group of **7a,b** yielded the 10-alkylpteroic acids, which were coupled with diethyl glutamate, hydrogenated over PtO_2, and saponified to afford 10-alkyl-5,10-dideazatetrahydrofolic acids **13a,b**. Aminopterin analogues **9a,b** were effective inhibitors of DHFR derived from L1210, but were less potent than methotrexate for inhibition of growth of L1210 in culture. The 10-ethyl (**13b**) analogue of 5,10-DDTHF was about twice as potent an inhibitor of L1210 cell growth as 5,10-DDTHF, but was only $^1/_7$ as potent for inhibition of GAR formyltransferase. 10-Methyl analogue **13a** was similar in potency to 5,10-DDTHF. All of the compounds showed moderately improved transport into L1210 cells relative to methotrexate.

Our previous studies of the antifolate and transport properties of deaza analogues of methotrexate have been mainly concerned with compounds in the pteridine and 8-deazapteridine series.[1,2] 10-Deazaminopterin and its 10-ethyl analogue have been introduced into clinical trials and showed interesting properties as antitumor agents.[3-6] 10-Alkyl-10-deaza analogues in these series have been shown to retain antifolate activity and exhibit enhanced transport into a variety of tumor cells compared with normal cells. It was also of interest to evaluate the 5,10-dideazaminopterin series in similar studies. We have recently communicated[7] the synthesis of 5,10-dideazaminopterin as have Taylor and co-workers[8] by an alternate procedure. In this manuscript we report the synthesis and activity of the 10-methyl (**9a**) and 10-ethyl (**9b**) analogues of 5,10-dideazaminopterin.

Beardsley et al.[9,10] have recently found that 5,10-dideazatetrahydrofolic acid (DDTHF) was also a potent antitumor agent whose locus of action entailed inhibition of glycinamide ribotide (GAR) formyltransferase. We have prepared the 10-methyl (**13a**) and 10-ethyl (**13b**) analogues of DDTHF and compared their relative abilities to inhibit GAR formyltransferase. In addition, we evaluated these compounds as inhibitors of aminoimidazolecarboxamide ribotide (AICAR) formyltransferase, another key enzyme in purine biosynthesis.

Chemistry

The synthetic procedure (Scheme I) used for preparation of **9a,b** was a modification of our method reported earlier for 5,10-dideazaminopterin itself.[7] *p*-Ethylbenzoic acid was converted in situ to its dianion and alkylated with 3-methoxyallyl chloride to afford the enol ether acid (**1a**) as reported earlier.[1] The acid was esterified via treatment of the sodium salt with methyl iodide in DMF to afford **2a** in a 27% overall yield from *p*-ethylbenzoic acid. Hydrolysis of enol ether methyl ester **2a** in 1 N HCl–HOAc gave aldehyde ester **3a** in 95% yield. The aldehyde was converted in 94% yield to the enamine by reaction with piperidine–K_2CO_3 and crude enamine **4a** was immediately condensed with (acetoxymethylene)malononitrile to afford the piperidino dienylmalononitrile intermediate (**5a**) in 37% yield. (Acetoxymethylene)malononitrile was conveniently prepared by acetylation[11] of the potassium salt of formylmalononitrile[12] with acetyl chloride. This reagent was much superior to (methoxymethylene)malononitrile which afforded yields of only 5–10% in similar condensations.[7]

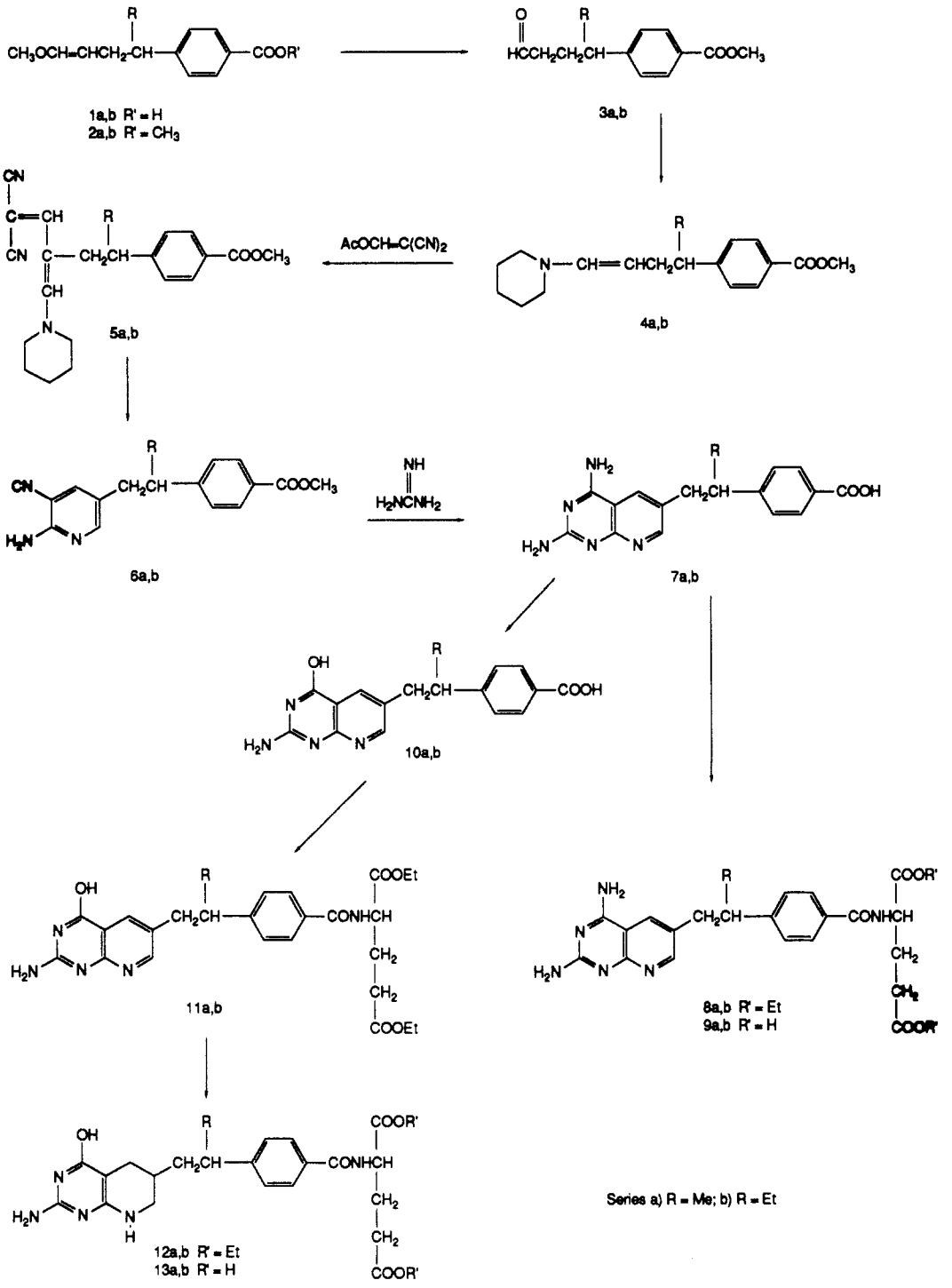
When **5a** was allowed to react with concentrated NH_4OH in MeOH at room temperature for 72 h, ring closure to 2-amino-3-cyanopyridine **6a** took place in 91% yield. Treatment of **6a** with 4 equiv of guanidine in 2-methoxyethanol at 115–120 °C for 24 h effected ring closure to the 5-deazapteridine, while addition of a little H_2O to the hot medium also caused hydrolysis of the benzoate ester to afford 4-amino-4-deoxy-5-deazapteroic acid (**7a**). Acid **7a** was used to acylate diethyl L-glutamate via the mixed anhydride process to yield diester **8a**. Following purification by chromatography on silica gel the diester was saponified with 1 N NaOH in 2-methoxyethanol to afford the target 10-methyl-5,10-dideazaminopterin (**9a**). 10-

† SRI International.
‡ Tufts University Health Sciences Campus.
§ Memorial Sloan Kettering Cancer Center.

(1) DeGraw, J. I.; Brown, V. H.; Tagawa, H.; Kisliuk, R. L.; Gaumont, Y.; Sirotnak, F. M. *J. Med. Chem.* **1982**, *25*, 1227.
(2) DeGraw, J. I.; Christie, P. H.; Brown, E. G.; Kelly, L. F.; Kisliuk, R. L.; Gaumont, Y.; Sirotnak, F. M. *J. Med. Chem.* **1984**, *27*, 376.
(3) Currie, V.; Warrell, R. P.; Arlin, Z.; Tan, C.; Sirotnak, F.; Green, G.; Young, C. *Cancer Treat. Rep.* **1983**, *67*, 149.
(4) Ahmed, T.; Yagoda, A.; Scher, H. I.; Sternberg, C.; Watson, R. C. *Invest. New Drugs* **1986**, *4*, 171.
(5) Kris, M. G.; Kinahan, J. J.; Gralla, R. J.; Fanucchi, M. P.; Wetheim, M. S.; O'Connell, J. P.; Marks, L. D.; Williams, L.; Farag, F.; Young, C. W.; Sirotnak, F. M. *Cancer Res.* **1988**, *48*, 5570.
(6) Shum, K. Y.; Kris, M. G.; Gralla, R. J.; Burke, M. T.; Marks, L. D.; Heelan, R. T. *J. Clin. Oncol.* **1988**, *6*, 446.
(7) DeGraw, J. I.; Tagawa, H.; Christie, P. H.; Lawson, J. A.; Brown, E. G. *J. Heterocycl. Chem.* **1986**, *23*, 1.
(8) Taylor, E. C.; Harrington, P. J.; Fletcher, S. R.; Beardsley, G. P.; Moran, R. G. *J. Med. Chem.* **1985**, *28*, 914.
(9) Beardsley, G. P.; Taylor, E. C.; Grindey, G. B.; Moran, R. G. *Chemistry and Biology of Pteridines*; Cooper, B. A., Whitehead, V. M., Eds.; Walter de Gruyter: Berlin, 1986; p 953.
(10) (a) Beardsley, G. P.; Taylor, E. C.; Shih, C.; Poore, G. A.; Grindey, G. B.; Moran, R. G. *Proc. Am. Assoc. Cancer Res.* **1986**, *27*, 259. (b) Moran, R. G.; Taylor, E. C.; Beardsley, G. P. *Proc. Am. Assoc. Cancer Res.* **1987**, *28*, 274.
(11) Rieser, J.; Friedrich, K. *Liebigs. Ann. Chem.* **1976**, 648.
(12) Josey, A. D.; Dickinson, C. L.; Dewhirst, K. C.; McKusick, B. C. *J. Org. Chem.* **1967**, *32*, 1941.

0022-2623/90/1833-0673$02.50/0

Scheme I



Ethyl analogue **9b** was similarly prepared by the above route beginning with *p*-propylbenzoic acid.

Treatment of diamino acid **7a** with hot 10% NaOH gave 10-methyl-5,10-dideazapteroic acid **10a**. This intermediate was coupled with diethyl L-glutamate as above and the resulting diester **11a** was hydrogenated over platinum oxide in ethanol containing 1 equiv of CF_3COOH. Tetrahydro diester **12a** was then saponified to yield 10-methyl-5,10-dideazatetrahydrofolic acid (**13a**). 10-Ethyl analogue **13b** was similarly obtained from the 4-amino-4-deoxy-10-ethyl-5,10-dideazapteroic acid intermediate (**7b**).

Biological Results

In Table I, data are presented that show the relative potencies of the 5,10-dideazaminopterin analogues **9a** and **9b** compared with that of methotrexate for inhibition of growth in L1210 leukemia cell culture. In addition the compounds were evaluated as inhibitors of dihydrofolate reductase derived from L1210 cells. The 10-methyl analogue (**9a**) was only $^1/_6$ as potent a growth inhibitor as MTX, while the 10-ethyl compound **9b** was $^1/_3$ as potent. However, **9a** was nearly equal to MTX for inhibition of DHFR, but the K_i for **9b** was about twice that of MTX. Compounds **9a** and **9b** were transported slightly better than MTX into L1210 cells as shown by the relative influx values. These results did not suggest that **9a** or **9b** would be greatly superior to MTX or its 10-deaza[1] or 8,10-dideaza[2] analogues.

The 10-methyl (**13a**) and 10-ethyl (**13b**) analogues of 5,10-DDTHF were moderate inhibitors of cell growth in

Table I. Cell Growth and Enzyme Inhibition[d-f]

compd	L1210 growth inhibn: IC_{50},[a] nM	L1210 DHFR inhibn: K_i,[a] nm	L1210 GAR formyl transferase:[c] IC_{50}, nM	*L. casei* GAR formyl transferase:[c] IC_{50}, nM	L1210 relative influx[b]
9a	23.9	0.007			1.43
9b	12.1	0.013			1.32
MTX	3.92	0.006			1.0
13a	50.3	>1000	1500	17	1.72
13b	34.6	>1000	660	80	1.36
5,10-DDTHF	57.2	>1000	500	10	1.54

[a] See ref 1 for methods. [b] Ratio vs MTX. [c] Assayed by the method of Smith et al., ref 16. [d] *L. casei* growth inhibition (IC_{50}, nM) **13a** (40), **13b** (80), 5,10-DDTHF (14). [e] AICAR formyltransferase (*L. casei*) inhibition for **13a**, **13b**, and 5,10-DDTHF was $IC_{50} > 10^{-5}$ M. [f] Thymidylate synthase (*L. casei*) inhibition for **13a**, **13b**, and 5,10-DDTHF was $IC_{50} > 10^{-4}$ M.

L1210 cultures. Compound **13a** was slightly more active than 5,10-DDTHF, while **13b** was 1.7 times as potent (Table I). All of these compounds were 1 order of magnitude less potent than MTX as inhibitors of L1210 growth. None of the tetrahydrofolate analogues were effective inhibitors of L1210 DHFR (Table I) or *Lactobacillus casei* thymidylate synthase ($IC_{50} > 10^{-4}$ M).

The inhibition of L1210 GAR formyltransferase was similar for all three tetrahydrofolate analogues. 5,10-DDTHF was 3 times as effective as **13a** and 1.3 times as effective as **13b** (Table I). These small differences in enzyme inhibition did not correlate with the small differences in L1210 cell growth inhibition noted above. All three tetrahydrofolate analogues were much more potent (8–88-fold) as inhibitors of *L. casei* GAR formyltransferase as compared with the L1210 enzyme. For *L. casei*, GAR formyltransferase inhibition (Table I) correlates roughly with *L. casei* growth inhibition where the IC_{50} values for **13a**, **13b**, and 5,10-DDTHF are 40, 80, and 14 nM, respectively. Compounds **13a**, **13b**, and 5,10-DDTHF were ineffective as inhibitors of L1210 or *L. casei* AICAR formyltransferases ($IC_{50} > 10^{-5}$ M).

The tetrahydro analogues evaluated in these studies were diastereomeric about the 6- and 10-positions. It is possible that the pure diastereomers differ with regard to their inhibitory properties. However, our previous studies with pure diastereomers of 10-methyl- and 10-ethyl-10-deazaminopterin,[13] showed little differences for in vitro and in vivo inhibitory and transport properties among the isomers. Our work tends to confirm that the antitumor results with 5,10-DDTHF observed by Beardsley et al.[9,10] are related to the inhibition of GAR formyltransferase. Since these compounds are most likely converted to polyglutamated species intracellularly, it is quite possible that a particular polyglutamated form may be ultimately responsible for the in vivo activity. These analogues of tetrahydrofolate were favorably transported into L1210 cells relative to MTX (Table I) but the differences in influx between analogues are small. Further comparisons of these tetrahydrofolate analogues in L1210-sensitive and -resistant cell lines will be published elsewhere.[15]

Experimental Section

Elemental analyses were obtained from Galbraith Laboratories, Knoxville, TN. Values were within 0.4% except as noted in parenthesis following the element. The ^{1}H NMR spectra were taken on a Varian 360A or a JEOL FX90Q spectrometer. Mass spectra were run on a LKB 9000 GC-MS spectrometer or a Ribermag R10-10C MS system. Ultraviolet spectra were taken on a Perkin-Elmer 552 or Perkin-Elmer-Coleman 575. Melting points were determined on a Thomas-Hoover Uni-melt apparatus.

1-Methoxy-4-(*p*-carbomethoxy)phenyl-1-pentene (2a). To a solution of 84 mL (0.60 mol) of freshly distilled diisopropylamine in 765 mL of dry THF at 0–5 °C under argon was added 419 mL (0.63 mol) of 1.5 M *n*-BuLi in hexane over 30 min. After another 30 min a solution of 45.0 g (0.30 mol) of *p*-ethylbenzoic acid in 162 mL of THF was added over 30 min followed by addition of 53 mL of HMPA. The mixture was stirred at 0–5 °C for 6 h and then at ambient temperature for 22 h. The dark red solution was cooled to 0–5 °C and treated dropwise with 1.35 M 3-methoxyallyl chloride in ether[14] until the color was quenched (230 mL, 77% consumed). After the addition of 5 mL of water the mixture was evaporated in vacuo. The residue was partitioned between 500 mL of water and 200 mL of $CHCl_3$–2-PrOH (9:1). The aqueous portion was washed with three additional 100-mL portions of $CHCl_3$–2-PrOH, chilled in ice, and acidified with HOAc to give an oily precipitate. The mixture was extracted with three 200-mL portions of CH_2Cl_2, and the combined extracts were washed with 150 mL of water, dried over $MgSO_4$, and evaporated to leave 59.1 g of crude enol ether acid **1a**.

A mixture of **1a** (59.1 g), 47.3 g of $NaHCO_3$, 35 mL of methyl iodide, and 270 mL of DMF was stirred at room temperature for 72 h. After dilution with 1300 mL of water the mixture was extracted three times with 300-mL portions of pentane. The pentane extract was washed with 200 mL of water, dried over $MgSO_4$, and evaporated in vacuo to leave 44.5 g of a brown oil. The oil was fractionated through a short Vigreux column to give 7.5 g of methyl *p*-ethylbenzoate, [bp 72 °C (0.5 mm)] and 18.8 g (27%) of product [bp 126–128 °C (0.5 mm)]: NMR ($CDCl_3$) δ 1.23 (3 H, d, $CHCH_3$), 2.0–3.1 (3 H, m, CH_2CHCH_3), 3.43 (3 H, s, OCH_3), 3.90 (3 H, s, $COOCH_3$), 4.6 (1 H, m, 2-CH=), 6.3 (1 H, d, 1-CH=), 7.3 (2 H, d, 3′,5′-ArH), and 8.07 (2 H, d, 2′,6′-ArH).

Ethyl homologue **2b** was similarly prepared from *p*-propylbenzoic acid in 28% yield: bp 145–147 °C (0.7 mm); NMR ($CDCl_3$) δ 0.77 (3 H, t, CH_2CH_3), 1.4–2.8 (5 H, m, $CH_2CH_3 + CH_2CH$–), 3.42 (3 H, s, OCH_3), 3.9 (3 H, s, $COOCH_3$), 4.60 (1 H, m, 2-CH=), 6.25 (1 H, d, 1-CH=), 7.27 (2 H, d, 3′,5′-ArH), and 8.05 (2 H, d, 2′,6′-ArH). Anal. ($C_{15}H_{20}O_3$) C, H.

(Acetoxymethylene)malononitrile. The potassium enolate salt of formylmalononitrile[12] (36.0 g, 0.27 mol) was suspended in 240 mL of dry CH_2Cl_2 and treated dropwise with 19.2 mL (0.27 mole) of acetyl chloride. The mixture was stirred at reflux for 3 h and cooled, and the KCl was removed by filtration. The filtrate was evaporated and the residual liquid was distilled in vacuo to afford 31.3 g (85%): bp 88–91 °C (0.5 mm); NMR ($CDCl_3$) δ 2.43 (3 H, s, $COCH_3$), 8.6 (1 H, s, =CH).

4-(*p*-Carbomethoxyphenyl)valeraldehyde (3a). Enol ether ester **2a** (12.8 g), 144 mL of acetic acid, and 19.5 mL of 1 N HCl were stirred at room temperature for 18 h. The solvent was evaporated in vacuo and the residual oil was diluted with 150 mL of water. The product was extracted into three 75-mL portions of ether and the extract was washed with 100 mL of saturated $NaHCO_3$, dried over $MgSO_4$, and evaporated to leave 11.5 g (95%) of aldehyde: NMR ($CDCl_3$) δ 1.3 (3 H, d, $CHCH_3$), 1.7–3.0 (5 H, m, $-CH_2CH_2CH$–), 3.9 (3 H, s, $COOCH_3$), 7.3 (2 H, d, 3′,5′-ArH), 8.07 (2 H, d, 2′,6′-ArH), and 9.82 (1 H, s, CHO).

4-(*p*-Carbomethoxyphenyl)hexanal (3b) was similarly obtained from **2b** in 97% yield: NMR ($CDCl_3$) δ 0.77 (3 H, t,

(13) DeGraw, J. I.; Christie, P. H.; Tagawa, H.; Kisliuk, R. L.; Gaumont, Y.; Schmid, F. A.; Sirotnak, F. M. *J. Med. Chem.* **1986**, *29*, 1056.
(14) Hoff, S.; Brandtsma, L. *Recl. Trav. Chim. Pays-Bas.* **1969**, *88*, 845.
(15) Sirotnak, F. M.; Otter, G. M.; Piper, J. R.; DeGraw, J. I. *Biochem. Phamacol.* In press.
(16) Smith, G. K., Benkovic, P. A.; Benkovic, S. J. *Biochemistry* **1981**, *20*, 4034.

CH_2CH_3), 1.2–2.8 (7 H, m, $CH_2CH_2CHCH_2$), 3.9 (3 H, s, $COOCH_3$), 7.27 (2 H, d, 3′,5′-ArH), 8.05 (2 H, d, 2′,6′-ArH), and 9.77 (1 H, s, CHO).

1,1-Dicyano-3-(piperidinomethylene)-5-(*p*-carbomethoxyphenyl)hex-1-ene (5a). To 1.27 g (5.78 mmol) of aldehyde **3a** was added dropwise 0.68 mL (6.86 mmol) of piperidine with cooling by a cold-water bath. Powdered K_2CO_3 (2.1 g, 15 mmol) was added and the mixture was stirred at 60 °C (preheated bath) for 40 min. The mixture was cooled, extracted with three 10-mL portions of CH_2Cl_2, and filtered rapidly, and the filtrate was evaporated in vacuo to leave 1.63 g (94%) of enamine **4a**: NMR ($CDCl_3$) δ 3.90 (3 H, s, $COOCH_3$), 5.20 (1 H, m, 2-CH=), and 5.83 (1 H, d, 1-CH=), 7.3 (3′,5′-ArH), and 8.05 (2′,6′-ArH).

Enamine (1.63 g, 5.41 mmol) was dissolved in 25 mL of THF and treated with 0.78 mL (5.6 mmol) of Et_3N. The solution was cooled to −20 °C and a solution of 0.63 g (5.6 mmol) of (acetoxymethylene)malononitrile in 7 mL of THF was added dropwise. The mixture was kept at ambient temperature for 15 h and filtered to remove Et_3NHOAc, and the filtrate was evaporated to dryness. The oily residue was washed with 10 mL of Et_2O, taken up in 3 mL of MeCN, and chilled. The orange-yellow precipitate was collected to afford 372 mg, mp 162–165 °C. The mother liquor was chromatographed on silica gel with elution by $CHCl_3$–EtOAc (97.5:2.5) to yield another 409 mg of product: total yield 781 mg (37%) from aldehyde **3a**; NMR ($CDCl_3$) δ 1.37 (3 H, d, CH_3), 1.63 (6 H, br s, 3,4,5-piperidino), 2.5–3.6 (7 H, m, 2,6-piperidino + CH_2CH), 3.93 (3 H, s, $COOCH_3$), 6.72 (1 H, s, NCH=), 6.90 (1 H, s, $CH=C(CN)_2$), 7.33 (2 H, d, 3′,5′-ArH), 8.03 (2 H, d, 2′,6′-ArH); UV_{max} (EtOH) 240, 377 nm. An analytical sample was obtained from EtOH, mp 163–165 °C. Anal. ($C_{22}H_{25}N_3O_2$) C, H, N.

The corresponding ethyl homologue (**5b**) was similarly prepared from aldehyde (**3b**) in 54% yield: mp 158–161 °C (EtOH); NMR ($CDCl_3$) δ 0.8 (3 H, t, CH_3), 1.73 (6 H, br s, 3,4,5-piperidino), 2.5–3.7 (9 H, m, 2,6-piperidino, CH_2CHCH_2), 3.93 (3 H, s, $COOCH_3$), 6.77 (1 H, s, NCH=), 6.87 (1 H, s, CH=C), 7.33 (2 H, d, 3′,5′-ArH), 8.06 (2 H, d, 2′,6′-ArH). Anal. ($C_{23}H_{27}N_3O_2$) C, H, N.

1-(2′-Amino-3′-cyano-5′-pyridyl)-2-(*p*-carbomethoxyphenyl)propane (6a). Enamino dinitrile **5a** (5.04 g, 13.87 mmol) was suspended in 154 mL of MeOH and treated with 6.4 mL of concentrated NH_4OH. The mixture was stirred at room temperature for 72 h to afford a solution whose high-wavelength absorption showed a maximum at 333 nm compared with 377 nm observed for **5a**. The solvent was evaporated in vacuo and the residue was triturated with 50 mL of MeOH. The crystals were collected, washed with MeOH, and dried to leave 3.22 g, mp 134–135 °C. Concentration of the filtrate to a volume of 10 mL afforded another 0.52 g (mp 132–134 °C): total yield 3.74 g (91%); UV_{max} (EtOH) 274, 333 nm; NMR ($CDCl_3$) δ 1.3 (3 H, d, CH_3), 2.7–3.5 (3 H, m, CH_2CH), 3.92 (3 H, s, $COOCH_3$), 5.17 (2 H, s, NH_2), 7.3 (3 H, m, 3′,5′-ArH and C-6H), 8.03 (d, 3 H, 2′.6′-ArH and C-4H). Anal. Calcd for $C_{17}H_{17}N_3O_2$: C, H, N.

Ethyl homologue **6b** was obtained in a similar manner from **5b** in 73% yield. An analytical sample, mp 95.5–97.5 °C, was obtained as white crystals from 2-PrOH: NMR ($CDCl_3$) δ 0.75 (3 H, t, CH_3), 1.64 (2 H, q, $-CH_2CH_3$), 2.72 (3 H, m, CH_2CHAr), 3.87 (3 H, s, $COOCH_3$), 5.23 (2 H, s, NH_2), 7.07 (d, 3′,5′-ArH), 7.23 (1 H, d, C-6H), 7.7 (1 H, d, C-4H), 7.9 (2 H, d, 2′,6′-ArH). Anal. Calcd for $C_{18}H_{19}N_3O_2$: C, H, N.

4-Amino-4-deoxy-10-methyl-5,10-dideazapteroic Acid (7a). To a solution of metallic Na (1.17 g, 0.051 g-atom) in 205 mL of 2-methoxyethanol was added 4.85 g (0.051 mol) of guanidine hydrochloride. The mixture was stirred at room temperature for 10 min and 3.75 g (0.013 mol) of the amino cyanopyridine ester (**6a**) was added. The resulting solution was stirred at 115–120 °C for 24 h, whereupon the UV spectrum (EtOH) showed a shift of the longwave maximum to 347 nm from 333 nm. Water (2.0 mL) was added and heating continued at 100 °C for another 4 h. The solvent was evaporated in vacuo and the residue was treated with 200 mL of H_2O. Upon standing for 15 min, some insoluble material was removed by filtration. The filtrate was acidified with HOAc to pH 5–6 and the precipitate was collected, washed with H_2O, and dried to leave 3.33 g (81%) of product: UV_{max} (pH 13) 225 (ε 25858), 239 (ε 24607), 345 nm (ε 5614); MS *m/e* tris(trimethylsilyl) derivative, 539; NMR (DMSO-d_6) δ 1.22 (3 H, d, CH_3), 7.3 (2 H, d, 3′,5′-ArH), 7.87 (3 H, d, 2′,6′-ArH + 5-H), and 8.35 (1 H, s, 7-H). An analytical sample was obtained by reprecipitation from dilute NH_4OH. Anal. Calcd for $C_{17}H_{17}N_5O_2 \cdot {}^1/_2H_2O$: C, H (calcd 5.46, found 5.91), N (calcd 21.1, found 21.6).

10-Et homologue **7b** was obtained in a similar manner from **6b** in 67% yield; UV_{max} (pH 13) 227 (ε 24854), 241 (ε 25084), 343 nm (ε 5400); MS *m/e* 337 ($C_{18}H_{23}N_5O_2$). A satisfactory elemental analysis was not obtained and the material was used in the following step.

10-Methyl-5,10-dideazaminopterin Diethyl Ester (8a). To a stirred suspension of 600 mg (1.86 mmol) of 4-aminopteroic acid **7a** in 11 mL of dry DMSO was slowly added 0.39 mL (2.8 mmol) of Et_3N and 0.36 mL (2.8 mmol) of isobutyl chloroformate. A complete solution resulted which was kept at room temperature for 1 h. Then, 0.42 mL (3.03 mmol) of Et_3N and 723 mg (3.03 mmol) of diethyl L-glutamate hydrochloride were added. The mixture was stirred for 1.5 h and two additional courses of the Et_3N–*i*-BuOCOCl–ethyl glutamate reagents were administered in the same manner and the final mixture stirred at room temperature for 18 h. Ice water (100 mL) was added and the mixture was extracted three times with 15-mL portions of $CHCl_3$. The extract was washed with 20 mL of saturated $NaHCO_3$, dried over $MgSO_4$, and evaporated to dryness to leave 0.58 g. The crude material was chromatographed on 20 g of silica gel with initial elution by $CHCl_3$, followed by $CHCl_3$–MeOH (97.5:2.5) to afford 166 mg (18%) of off-white solid. A sample was recrystallized from MeOH–Et_2O for analysis: mp 213–215 °C; MS *m/e* 508; NMR ($CDCl_3$ + CD_3OD) δ 1.23 (9 H, m, CH_3), 2.4 (4 H, m, $-CH_2CH_2-$), 2.9 (3 H, m, 9,10-CH_2CH), 4.13 (4 H, q, OCH_2), 4.77 (1 H, d, NHC*H*), 7.2 (2 H, d, 3′,5′-ArH), 7.8 (2 H, d, 2′,6′-ArH), 8.0 (1 H, s, 5-H), 8.43 (1 H, s, 7-H). Anal. Calcd for $C_{26}H_{32}N_6O_5 \cdot {}^3/_4H_2O$: C, H, N.

10-Ethyl analogue **8b** was similarly obtained from **7b** in an 18% yield: mp 153–156 °C; MS *m/e* 522. Anal. Calcd for $C_{27}H_{34}N_6O_5 \cdot 2H_2O$: C, H. N.

10-Methyl-5,10-dideazaminopterin (9a). A solution of 123 mg of diester **8a** in 1.7 mL of 2-methoxyethanol was treated with 1.7 L of 1 N NaOH, and the resulting solution was kept at room temperature for 4 h. Water (1.7 mL) was added and the pH was adjusted to 7 with HOAc. The solution was evaporated to dryness in vacuo and the residue was taken up in 4 mL of H_2O. After acidification to pH 5–6 with HOAc, the white precipitate was collected, washed with H_2O, and dried to leave 80 mg (73%): HPLC indicated 94% purity; UV_{max} (pH 13) 226 (ε 30072), 246 (ε 29217), 347 nm (ε 6185). Anal. Calcd for $C_{22}H_{24}N_6O_5 \cdot 2H_2O$: C, H, N.

10-Ethyl analogue **9b** was similarly prepared in 74% yield: UV_{max} (pH 13) 226 (ε 29772), 246 (ε, 28644), 346 nm (ε 5751); NMR (DMSO-d_6) δ 0.73 (3 H, t, CH_3), 1.70 (2 H, m, CH_2CH_3), 2.05 (2 H, m, CH_2-glutamate), 2.35 (2 H, m, CH_2COOH), 2.95 (3 H, m, 9-CH_2, 10-H), 4.38 (1 H, m, C*H*NH), 7.27 (2 H, d, 3′,5′-ArH), 7.80 (3 H, d, 2′,6′-Ar, 5-H), 8.25 (1 H, s, 7-H). Anal. Calcd for $C_{23}H_{26}N_6O_5 \cdot 1.5H_2O$: C, H. N.

10-Methyl-5,10-dideazapteroic Acid (10a). A solution of 2 g, (6.2 mmol) of diamino acid **7a** in 38 mL of 10% NaOH was heated at reflux for 2.5 h (UV shifted from 347 to 335 nm). The solution was cooled and acidified with 6 N HCl to cause precipitation of the product. The precipitate was collected, washed with H_2O, and dried to leave 2 g (100%): UV_{max} (pH 13) 242 (ε 27505), 337 nm (ε 5994); MS *m/e* 324 ($C_{17}H_{16}N_4O_3$). Elemental analysis indicated a hydrated partial HCl salt. The material was used directly in the next step.

10-Ethyl-5,10-dideazapteroid acid (10b) was obtained from **7b** in 94% yield via a similar procedure; UV_{max} (pH 13) 241 (ε 30770), 335 nm (ε 6697). A satisfactory elemental analysis was not obtained and the material was used directly in the following step.

10-Methyl-5,6,7,8-tetrahydro-5,10-dideazafolic Acid (13a). To a solution of 1.50 g (4.62 mmol) of 10-methylpteroic acid **10a** in 26.5 mL of dry DMSO was added 1.29 mL (9.24 mmol) of Et_3N followed by dropwise addition of 1.20 mL (9.24 mmol) of isobutyl chloroformate. The mixture was stirred for 1 h at room temperature when another 1.29 mL (9.24 mmol) of Et_3N and 2.19 g (9.24 mmol) of diethyl L-glutamate were added. The resulting mixture was stirred for 20 h at ambient temperature and diluted with 220 mL of ice water. The pale yellow precipitate was collected

by filtration and thoroughly washed with water and Et_2O. The solid was then stirred with 50 mL of saturated $NaHCO_3$, followed by filtration. The filter cake was washed with water and dried to leave 1.59 g. The solid was twice extracted with hot (100 °C) DMF and the solvent was evaporated in vacuo to leave 0.96 g (41%) of pale yellow solid diester **11a**; MS *m/e* 509 ($C_{26}H_{31}N_5O_6$).

A suspension of **11a** (609 mg, 1.20 mmol) in 22 mL of EtOH was treated with 1.74 mL (22.6 mmol) of CF_3COOH to give a solution. PtO_2 (890 mg) was added and the mixture was stirred under 1 atm of H_2 for 44 h. The catalyst was removed by filtration through Celite and the filtrate was evaporated to dryness. The residue was dissolved in 30 mL of $CHCl_3$, washed with 15 mL of saturated $NaHCO_3$, and dried over $MgSO_4$, and the $CHCl_3$ was evaporated to leave 0.43 g of yellow gum. The material was chromatographed on 14 g of silica gel with elution by $CHCl_3$–MeOH (95:5) to afford 0.24 g of **12a** as a pale yellow semisolid; MS *m/e* 513 ($C_{26}H_{35}N_5O_6$).

The tetrahydro diester (225 mg) was treated with 10 mL of EtOH and 20 mL of 0.1 N NaOH and the solution was heated at 100 °C for 15 min. The solution was cooled, adjusted to pH 5 with HOAc, and evaporated in vacuo at 25 °C until precipitation of the product occurred. The precipitate was collected, washed with H_2O, and dried to leave 131 mg (66%) of **13a**; UV_{max} (pH 13) 240 (ε 19746), 269 nm (ε 11504); MS *m/e* 457; NMR (DMSO-d_6) δ 1.2 (3 H, d, CH_3), 1.45 (3 H, C6 + 9-CH_2), 2.0 (2 H, m, CH_2-glu), 2.3 (2 H, d, CH_2COOH) 2.8–3.3 (5 H, m, C-5-CH_2, 10-H, 7-CH_2), 4.36 (1 H, d, NHC*H*), 5.9 (2 H, s, NH_2), 7.3 (2 H, d, 3′,5′-ArH), 7.76 (2 H, d, 2′,6′-ArH), 8.45 (1 H, d, CONH). Anal. Calcd for $C_{22}H_{27}N_5O_6 \cdot 2H_2O$: C, H, N.

10-Ethyl-5,6,7,8-tetrahydro-5,10-dideazafolic acid (13b) was similarly obtained from **10b**. Crude diester **11b** was obtained in 39% yield: MS *m/e* 253 ($C_{27}H_{33}N_5O_6$). Hydrogenation over PtO_2 gave tetrahydro diester **12b** in 25% yield as the trifluoroacetate salt: mp 191–193 °C; MS *m/e* 527 ($C_{27}H_{37}N_5O_6$); NMR ($CDCl_3$) δ 0.73 (3 H, t, CH_3), 1.25 (6 H, m, ester CH_3), 1.65 (5 H, br s, 6-H, 9-CH_2, CH_2CH_3), 2.1–2.5 (4 H, m, $-CH_2CH_2-$), 2.5–3.3 (5 H, m, 5-CH_2, 10-H, 7-CH_2), 4.12 (4 H, q, $-OCH_2$), 4.75 (1 H, m, C*H*NH), 7.22 (2 H, d, 3′,5′-ArH), 7.78 (2 H, d, 2′,6′-ArH), 8.25 (1 H, br s, CONH). Anal. Calcd for $C_{27}H_{37}N_5O_6 \cdot CF_3COOH \cdot {}^1/_2H_2O$: C, H, N, F.

Saponification as above afforded **13b** in 59% yield: UV_{max} (pH 13) 240 (ε 19445), 271 nm (ε 11227). Anal. Calcd for $C_{23}H_{29}N_5O_6 \cdot 1.25H_2O$: C, H, N (calcd 14.2, found 13.7).

Acknowledgment. This work was supported by NIH Grants CA-28783 (J.I.D.), CA-18856 (F.M.S.), and CA-10914 (R.L.K.). We thank Dr. J. R. Piper for providing glycinamide ribonucleotide (GAR) and 5,10-DDTHF, and Dr. D. Thomas for mass spectrometric analyses.

Registry No. **1a**, 123751-61-1; **1b**, 123751-62-2; **2a**, 123751-63-3; **2b**, 123751-64-4; **3a**, 123751-65-5; **3b**, 123751-66-6; **4a**, 123751-67-7; **4b**, 123751-68-8; **5a**, 123751-69-9; **5b**, 123751-70-2; **6a**, 123751-71-3; **6b**, 123775-05-3; **7a**, 123751-72-4; **7b**, 123751-73-5; **8a**, 123751-78-0; **8b**, 123751-79-1; **9a**, 123751-82-6; **9b**, 123751-83-7; **10a**, 123751-74-6; **10b**, 123751-75-7; **11a**, 123751-76-8; **11b**, 123751-77-9; **12a**, 123751-80-4; **12b**, 123751-81-5; **13a**, 105580-37-8; **13b**, 105580-33-4; DHFR, 9002-03-3; GAR formyltransferase, 9032-02-4; 4-EtC_6H_4COOH, 619-64-7; 4-PrC_6H_4COOH, 2438-05-3; 3-MeOCH=CHCH_2Cl, 80986-54-5; $K^+ \cdot {}^-C(CN)_2CHO$, 118834-52-9; AcOCH=C$(CN)_2$, 86843-46-1; H-Glu(OEt)-OEt·HCl, 1118-89-4.

Novel Prodrugs Which Are Activated to Cytotoxic Alkylating Agents by Carboxypeptidase G2

Caroline J. Springer,*,† Pari Antoniw,† Kenneth D. Bagshawe,† Frances Searle,† Graham M. F. Bisset,‡ and Michael Jarman‡

Cancer Research Campaign Laboratories, Department of Medical Oncology, Charing Cross Hospital, London W6 8RF, England, and Drug Development Section, Institute of Cancer Research, Sutton, Surrey SM2 5NG, England. *Received June 8, 1989*

The synthesis of three novel prodrugs, 4-[bis[2-(mesyloxy)ethyl]amino]benzoyl-L-glutamic acid (**7**), 4-[(2-chloroethyl)[2-(mesyloxy)ethyl]amino]benzoyl-L-glutamic acid (**8**), and 4-[bis(2-chloroethyl)amino]benzoyl-L-glutamic acid (**9**), for use as anticancer agents, is described here. Each is a bifunctional alkylating agent in which the activating effect of the ionized carboxyl function is masked through an amide bond to the glutamic acid residue. These relatively inactive prodrugs are designed to be activated to their corresponding nitrogen alkylating agents (**10**, **11**, and **12**, respectively) at a tumor site by prior administration of a monoclonal antibody conjugated to the bacterial enzyme carboxypeptidase G2 (CPG2). The viability of two different tumor cell lines was monitored with each prodrug in the presence of CPG2. All three compounds showed substantial prodrug activity—with conversion to the corresponding active drug leading to greatly increased cytotoxicity.

Over the years, many cytotoxic compounds have been discovered which are of potential use in cancer chemotherapy.[1] Nitrogen mustards form one important family of such cytotoxic compounds.[2] The clinical effectiveness of cytotoxic compounds in general and nitrogen mustards in particular has been limited by the poor selectivity in the cytotoxic effect between tumor cells and normal cells. One approach to overcome this problem has involved the development of prodrugs which are potential precursors of the cytotoxic drug and whose cytotoxic properties are considerably reduced compared to those of the parent drug.[3]

Numerous proposals have been made for the administration of such prodrugs to patients under regimes whereby the prodrug is only converted into the cytotoxic drug in the region of the intended site of action.[4] However, such approaches typically entail activation by an organ (e.g. the liver) rich in drug-metabolizing enzymes in the hope that organ-derived tumor cells will be selectively killed.[5] It has rarely proved possible to achieve prodrug activation specifically at cancer sites because human cancers do not in general exhibit intrinsic metabolic properties sufficiently

* To whom all correspondence should be sent.
† Charing Cross Hospital.
‡ Institute of Cancer Research.

(1) Jarman, M. *Chem. Brit.* **1989**, *25*, 51.
(2) Ross, W. C. J. In *Biological Alkylating Agents*; Butterworth: London, 1962.
(3) Israels, L. G.; Ritzman, S. E. *Acta Unio Int. Cancrum* **1960**, *16*, 665.
(4) Bukhari, A.; Connors, T. A.; Gilsenan, A. M.; Ross, W. C. J.; Tisdale, M. J.; Warwick, G. P.; Wilman, D. E. V. *J. Natl. Cancer Inst.* **1973**, *50*, 243.
(5) Curt, G. A.; Connors, T. A.; Murray-Lyon, I. M.; Tattersall, M. H. N. *Biochem. Pharm.* **1977**, *26*, 1715.

0022-2623/90/1833-0677$02.50/0