Synthesis and Antitumor Activity of 10-Alkyl-10-deazaminopterins. A Convenient Synthesis of 10-Deazaminopterin

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Requirements for large-scale synthesis of the potent antitumor drug 10-deazaminopterin have led to development of a facile synthesis of this compound and its 10-alkyl analogues. The lithium diisopropyl amide generated dianions of appropriate *p*-alkylbenzoic acids were alkylated with 3-methoxyallyl chloride. The resulting 4-(*p*-carboxyphenyl)-1-methoxy-1-butenes were brominated at pH 7–8 to afford the 2-bromo-4-(*p*-carboxyphenyl)butyraldehydes. Condensation with 2,4,5,6-tetraminopyrimidine and subsequent in situ oxidation of the resulting dihydropteridines yielded crystalline 10-alkyl-10-deaza-4-amino-4-deoxypteroic acids. The pteroic acids were coupled with diethyl glutamate via the mixed anhydride method, followed by saponification at room temperature, to give the target 10-deazaminopterins. The 10-alkyl compounds were approximately equipotent to 10-deazaminopterin as growth inhibitors of folate-dependent bacteria. Their abilities to inhibit *Lactobacillus casei* and L1210 derived dihydrofolate reductases were also similar. Transport properties in vitro were suggestive of an improved therapeutic index for the 10-alkyl analogues. Against L1210 in mice, the percent increase in life span at the LD_{10} dosage was +151% (methotrexate), +178% (10-deazaminopterin), +235% (10-methyl analogue), and +211% (10-ethyl analogue). 10,10-Dimethyl-10-deazaminopterin was less effective at an equimolar dosage, but the ILS at the maximum dose tested (72 mg/kg) was +135%. It was far less toxic than the other analogues possibly because of enhanced clearance.

The antimicrobial and antileukemic activities of the potent dihydrofolate reductase inhibitors aminopterin and its N^{10}-methyl derivative, methotrexate (MTX), are well known. MTX has occupied a paramount position in the clinical management of certain leukemias for nearly 30 years. However, MTX has not found wide application for treatment of other forms of cancer. Its weak to marginal effects on human tumors is probably a reflection of its inability to readily penetrate the tumor tissues. We have reported the synthesis[1] and antimicrobial[1] and antitumor[2-4] effects in mice of 10-deazaminopterin (10-DA), an analogue in which the N^{10}-methylamino group of MTX is replaced by a methylene unit. The potent antitumor effect of 10-DA and its wide spectrum of activity have, in fact, led to initiation of clinical trials for this compound. Even though 10-DA was an effective drug, it was perceived that alkyl substitution about the ethylene bridge region could give compounds with enhanced differential transport in tumor vs. normal proliferative tissue.[14]

Chemistry. The need to supply larger amounts of 10-DA (**7a**) to support clinical investigation prompted a reinvestigation of its lengthy synthesis. An abbreviated route was developed, which also allowed the facile preparation of 10-alkyl substituted analogues of 10-DA.[5,6] The original synthetic process[1,7] for 10-DA was derived from a classical Boon–Leigh[8] type of ring closure for 6-substituted pteridines and afforded the key intermediate 4-amino-4-deoxy-10-deazapteroic acid (**5a**) in a 5% overall yield.

Our improved synthetic route is shown in Scheme I. Treatment of an ethereal solution of 1-methoxyallene (1) at −78 °C with an equivalent of hydrogen chloride in ether, followed by 24–48 h storage at 0–5 °C, affords 3-methoxyallyl chloride (**2**).[9] The unstable reagent was not isolated, but used in situ to alkylate the dianion of *p*-toluic acid as derived[10] by treatment with 2 equiv of lithium diisopropyl amide (LDA) at 0–5 °C in tetrahydrofuran. After the deep red color of the dianion was quenched, solvent was removed and the residue partitioned between ether and water. The aqueous portion was adjusted to pH 7–8, and the enol ether carboxylate (**3a**) was brominated with an equivalent of Br_2 in dichloromethane. Following acidification, the bromo aldehyde acid (**4a**) was removed by extraction and condensed with 2,4,5,6-tetraminopyrimidine in dilute acetic acid under conditions of the Waller reaction.[11] The dihydropteridine was not isolated but oxidized in situ with aqueous KI_3 to afford the crystalline 4-amino-4-deoxy-10-deazapteroic acid (**5a**). The overall yield was about 10% and the process could be conducted on a molar scale. After glutamate coupling and saponification, the 10-DA (**7a**) was obtained. Purification to levels of 98–99% was achieved by preparative reverse-phase liquid chromatography in a low-pressure apparatus. Thus, 10-g batches of crude 10-DA were chromatographed on 300 g of Bondapak C_{18}[12] with application as the ammonium salt and elution by water.

The synthetic process was also applied to preparation of the 10-methyl-(**7b**), 10-ethyl- (**7c**), and 10,10-dimethyl-10-deazaminopterin (**7d**) analogues. Anion formation with *p*-ethyl- or *p*-propylbenzoic acids was found to proceed considerably slower. These acids were allowed to react with LDA at room temperature for 15 h (or 24–30

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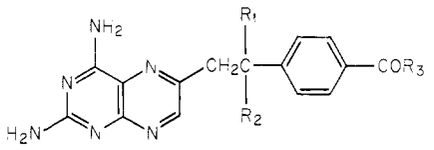
Table I. Physical Data for 10-Deazaminopterin Analogues



no.	R_1	R_2	R_3	yield, %	UV max, nm (ϵ), at pH 13	formula
5a	H	H	OH	12	253 (25 400)[a] 370 (6400)	
5b	CH_3	H	OH	32	255 (24 774) 369 (6741)	$C_{16}H_{16}N_6O_2 \cdot 1.5H_2O$
5c	C_2H_5	H	OH	32	255 (26 281) 370 (6398)	$C_{17}H_{18}N_6O_2 \cdot 0.4CH_3OH$
5d	CH_3	CH_3	OH	13	255 (26 492) 370 (6411)	$C_{17}H_{18}N_6O_2 \cdot 0.8H_2O$
7a	H	H	NHCH (Glu) CH_2CH_2COOH	61[d]	254 (33 170) 375 (7460)[b]	
7b	CH_3	H	Glu	38	255 (30 465) 371 (7433)	$C_{21}H_{23}N_7O_5 \cdot 1.75H_2O$
7c	C_2H_5	H	Glu	30	255 (30 731) 370 (7582)	$C_{22}H_{25}N_7O_5 \cdot 1.75H_2O$
7d	CH_3	CH_3	Glu	26	255 (29 583) 370 (7112)	$C_{22}H_{25}N_7O_5 \cdot 2.5H_2O$

[a] Reference 7 showed 256 nm (ϵ 22 200), 372 (5750). [b] Reference 1 showed 256 nm (ϵ 32 500), 372 (7475). [c] All new compounds were analyzed for C, H, and N within ±0.4% of theoretical values, except for the 10,10-Me_2 analogue. N: calcd 19.1; found, 18.6. [d] Yield from the pteroic acid.

Table II. Bacterial Growth and Enzyme Inhibition by 10-DA and Analogues

	concn, ng/mL,[a,c] for 50% inhibn of growth				concn, M, for 50% inhibn of	
	S. faecium		*L. casei*			
compd	ATCC 8043	MTX resist.	ATCC 7469	MTX resist.	dihydrofolate reductase[b,c]	thymidylate synthase[b,c]
5b	0.72	60	5.00	>2000	4.2×10^{-7}	3.9×10^{-4}
5c	0.90	50	5.40	>2000	3.8×10^{-7}	3.4×10^{-4}
5d	0.50	40	3.60	>2000	4.4×10^{-7}	4.0×10^{-4}
7a	0.20	>2000	0.01	>2000	2.0×10^{-8}	2.3×10^{-4}
7b	0.32	200	0.03	>2000	2.8×10^{-8}	2.8×10^{-4}
7c	0.37	1300	0.08	>2000	2.7×10^{-8}	3.3×10^{-4}
7d	0.25	>2000	0.04	>2000	2.7×10^{-8}	2.9×10^{-4}
MTX	0.05	3500	0.01	$>5 \times 10^5$	1.0×10^{-8}	0.8×10^{-4}

[a] Folate concentration = 1 ng/mL. [b] Enzyme derived from *L. casei*. [c] See ref 1 for conditions.

h at 0–5 °C) as contrasted with the 4–6 h at 0–5 °C required for *p*-toluic acid. *p*-Isopropylbenzoic acid could also be ionized but required about 24 h at room temperature with inclusion of hexamethylphosphoramide (HMPA) in the THF medium. The remainder of the process was analogous to that described above for 10-DA. The crystalline pteroic acid intermediates were coupled with diethyl glutamate, and the ester was saponified to afford the 10-alkyl-10-deazaminopterin analogues (**7b–d**). Purification was also accomplished by reverse-phase liquid chromatography on a small scale. However, on larger scales we experienced considerable losses of material due to irreversible absorption on reverse-phase columns. Consequently, it was found to be more profitable to chromatograph the diethyl esters (**6**) on conventional silica gel columns[13] prior to saponification to the target compounds.

Biological Results

In Table II, data are presented showing a comparison of relative potencies of compounds **5b–d** and **7a–d** vs. MTX with regard to growth inhibition of *Streptococcus faecium* and *Lactobacillus casei*. Also included are IC_{50} values for inhibition of the dihydrofolate reductase and thymidylate synthase derived from *L. casei*. In *S. faecium*, it is apparent that 10-alkyl substitution does not cause a great variation in potency as compared with 10-DA itself, except for the *S. faecium* MTX-resistant strain. In *L. casei*, a greater range of potencies was observed for the analogues (8-fold difference between **7a** and **7c**), but **7a–d** were all very potent. The pteroic acid compounds (**5b–d**) showed remarkable activity against the resistant strain of *S. faecium*. The strong inhibitory effects against dihydrofolate reductase observed for the analogues indicate a significant degree of bulk tolerance about the 10-position relative to acceptance at the active-site region of the reductase enzyme. As expected, the activity against thymidylate synthase was relatively low.

We have also investigated some biochemical and transport properties for these compounds in L1210 cells. In Table III, data are presented that show the effect of compounds **7a–d** as compared with MTX and aminopterin on dihydrofolate reductase, cell growth, and influx and efflux values.[14] All of the compounds had a similar ability to inhibit enzyme derived from L1210, with an average K_i of about 2.5×10^{-12} M. Compounds **7a–d** and aminopterin

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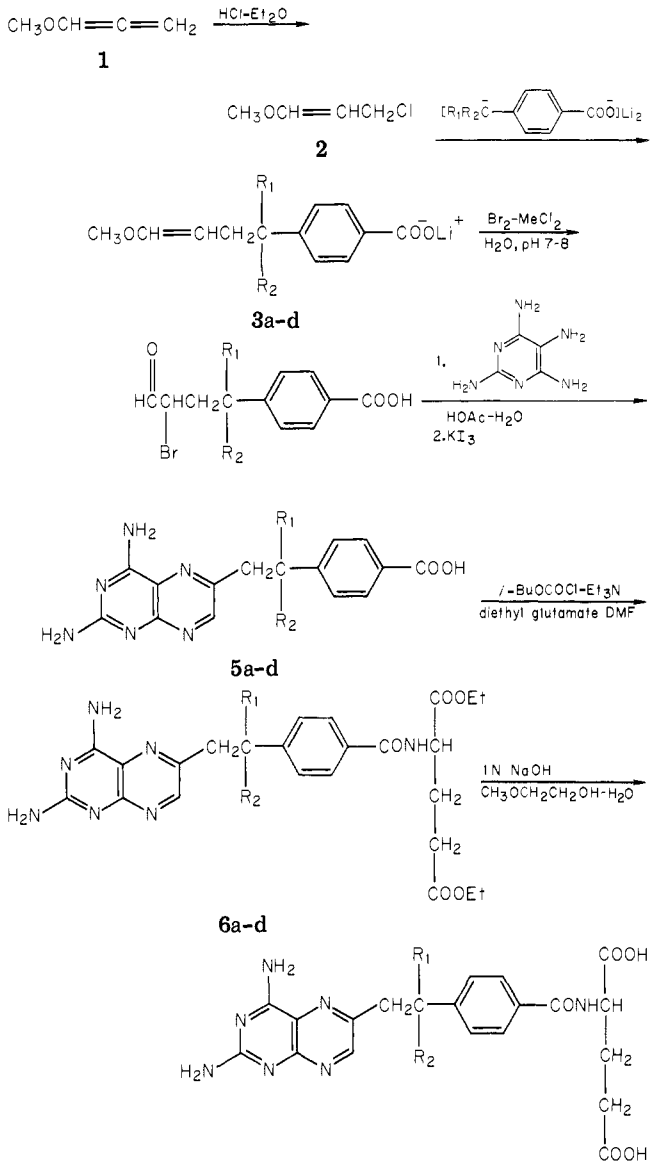
Table III. Biochemical and Growth Inhibition Data Derived with L1210 Cells[a]

compd	DHFR inhibn (K_i),[b] nM	growth inhibn (MIC_{50}),[c] nM	transport[b] influx K_m, μM	transport[b] influx v_{max}[d]	transport[b] efflux: K, min^{-1}
aminopterin	0.0023	0.61	1.21	7.83	0.21
MTX	0.0032	2.71	3.42	7.77	0.19
7a	0.0021	0.61	0.96	8.13	0.23
7b	0.0024	0.51	1.01	8.02	0.23
7c	0.0028	0.55	0.89	7.80	0.20
7d	0.0026	0.58	0.93	8.31	0.24

[a] Average of three to five runs; SE less than ±12%. [b] See ref 14 for methods. [c] See F. Sirotnak, P. Chello, D. Moccio, R. Kisliuk, G. Combepine, Y. Gaumont, and J. Montgomery, *Biochem. Pharmacol.*, **28**, 2993 (1979). [d] v_{max} = nanomoles per minute per gram of dry weight.

Scheme I



showed similar potencies for inhibition of growth of L1210 cells in culture, but MTX was only about one-fifth as potent.

The Michaelis constant (K_m) is a relative measure of the affinity for drug by the carrier component of the transport mechanism. It represents the external concentration of drug required for half-saturation and is one of the major kinetic determinants of responsiveness. Examination of the transport kinetics for compounds **7a–d** shows them to have approximately equivalent K_m values. Their affinity was significantly stronger than that of aminopterin but was very much stronger than for MTX. Influx velocities (v_{max}) were about equal for all of the drugs studied. The rate constants (k) for efflux were also quite similar. The effect of compounds **7a–d** on the toxicity limiting intestinal epithelial cells must also be determined before any potential therapeutic advantage may be assessed. Preliminary indications are that a substantial decrease in epithelial cell transport binding is obtained as the lipid content in the C_{9-10} bridge region is increased. This suggested that analogues **7b–d** would show an added therapeutic advantage over 10-DA (**7a**).

Table IV. Antitumor Effects against L1210 Leukemia in Mice

analogue[a]	e1d × 10[b] MST, days	e1d × 10[b] ILS, %	e2d × 5[c] MST, days	e2d × 5[c] ILS, %
control	6.6 ± 0.4		6.8 ± 0.3	
MTX	15.8 ± 0.9	+139	16.6 ± 0.5	+151
7a	17.3 ± 1.1	+162	18.4 ± 1.3	+178
7b	18.6 ± 1.3	+181	22.1 ± 1.7	+235
7c	17.8 ± 0.8	+170	20.5 ± 1.2	+211
7d	16.1 ± 1.4	+143	15.9 ± 1.6	+135

[a] Drug given sc 24 h after 10^6 cells implanted intraperitoneally; average of three experiments. [b] Dosages of 3 mg/kg for all analogues except **7d**, which was tested at the highest dose of 48 mg/kg, which is below the maximum tolerated dose. [c] LD_{10} dosages of 15 (MTX and **7a**) and 18 mg/kg (**7b** and **7c**); **7d** was tested at the highest dose of 72 mg/kg, which was blow the MTD.

In Table IV are shown the relative antitumor potencies of the compounds in murine L1210 leukemia. At the LD_{10} dosages, the 10-Me- and 10-Et-10-DA analogues were considerably more potent than 10-DA, which is itself more potent than MTX. There is a suggestion that optimal activity in this series is observed with the 10-methyl analogue. However, the effects in other tumor systems must be evaluated before reaching such a conclusion. The 10,10-dimethyl compound was significantly less active than the other compounds in the equimolar range. Since it is tolerated at a much higher dosage, it is quite likely that this agent is cleared more rapidly than the other analogues. It is interesting to note that 10-Me-10-DA and 10-Et-10-DA show lower acute toxicity than MTX or 10-DA in mice.

The observations reported in the above biological assays tend to confirm our hypothesis[2] that significant improvement in anticancer potential among antifolates can be achieved via modification of their cellular transport and in vivo distribution properties. The alterations in lipophilic character about the C_9–N_{10} bridge region of the folate skeleton were conducive to an improvement in rates of influx into tumor cells vs. drug-limiting intestinal ep-

ithelium. Since these structural modifications did not cause a significant decrease in inhibitory action against the target enzyme, dihydrofolate reductase, we were able to achieve a substantial amplification in antileukemic effect in vivo. Although it is too early in the clinical evaluation of 10-deazaminopterin to estimate its utility, it is likely that the 10-alkyl analogues will offer an even greater therapeutic advantage. Further evaluation of in vivo applications will be reported elsewhere.

Experimental Section

1-Methoxyallene (1) and 3-methoxyallyl chloride (2) were prepared by the procedure of Brandtsma.[9]

4-Amino-4-deoxy-10-deazapteroic Acid (5a). A solution of 192.8 mL (1.38 mol) of freshly distilled diisopropylamine in 1928 mL of dry THF was chilled to 0–5 °C, and a solution of 862 mL (1.38 mol) of 1.6 M butyllithium in hexane was added dropwise over 15 min under N_2. After 15 min a solution of 93.5 g (0.69 mol) of dry *p*-toluic acid in 385 mL of THF was added over 30 min at 0–5 °C. The dark red solution was tightly stoppered and maintained at 0–5 °C for 20 h. A solution of 0.83 M 3-methoxyallyl chloride in ether was added over 1 h at 0–5 °C and required 800 mL (96%) to quench the red color. The solvent was removed in vacuo and the residue was partitioned between 1 L of H_2O and 1 L of ether. The aqueous portion was washed with another 500 mL of ether and treated with CO_2 until a pH of 8–9 was obtained. Dichloromethane (240 mL) was added followed by the dropwise addition of 1 M Br_2 in CH_2Cl_2 at 0–5 °C until persistence of color (87% uptake of Br_2). Solid $NaHCO_3$ was added as necessary to maintain the pH at 7–8. The mixture was acidified to pH 1–2 with concentrated HCl, and the phases were separated. The aqueous portion was extracted with another 200 mL of CH_2Cl_2, and the combined organic extracts were dried over $MgSO_4$ and evaporated in vacuo to leave 180.2 g of crude semisolid bromo aldehyde acid (**4a**).

A mixture of 156.2 g (0.66 mol) of 2,4,5,6-tetraminopyrimidine sulfate, 160.3 g (0.65 mol) of $BaCl_2 \cdot 2H_2O$, and 3 L of H_2O was stirred at room temperature for 1.5 h. The mixture was warmed to 70 °C and filtered. The filtrate was cooled, adjusted to pH 3–4 with 10% NaOH, and warmed to 45 °C. A solution of the bromo aldehyde above in 1075 mL of acetic acid was added over 5 min, and the mixture was stirred at 45–50 °C for 1.5 h. After decantation from some insoluble gum, the supernatant containing the dihydropterin was treated dropwise with aqueous KI_3 (from 81.9 g of I_2, 156.2 g of KI, and 1075 mL of H_2O). Decolorization of the KI_3 ceased after addition of 700 mL. After 24 h at 0–5 °C, the yellow crystalline precipitate was collected, washed with H_2O and EtOH, and dried to leave 36 g. The crude material was stirred with 1800 mL of H_2O containing 20 mL of concentrated NH_4OH for 2 h and filtered. The filtrate was acidified with acetic acid to precipitate the product, which was collected, washed with H_2O, and dried to leave 25.5 g (12%): UV max at pH 13 230 nm (ϵ 22 100), 253 (25 400), 370 (6400); HPLC showed a single UV-absorbing peak (260 nm) in a CH_3OH–KH_2PO_4 buffer, pH 6 (25:75). The UV and HPLC were identical with authentic material prepared by our unequivocal synthesis.[7]

The 10-alkyl-10-deazapteroic acid analogues (**5b–d**) were prepared in a similar manner from the appropriate *p*-alkylbenzoic acids. The dianions from *p*-ethyl- and *p*-propylbenzoic acids were kept at room temperature for 15 h or at 0–5 °C for 24–30 h before alkylation with 3-methoxyallyl chloride. The dianion from *p*-isopropylbenzoic acid required the addition of 15% HMPA, with standing for 24 h to ensure complete formation. Analytical samples were obtained by recrystallization from methanol.

10-Deazaminopterin (7a). A mixture of 18.1 g (0.058 mol) of the pteroic acid (**5a**), 15.8 mL of triethylamine (0.116 mol), and 900 mL of dry DMF was warmed to 80 °C with stirring until nearly complete solution was obtained. The solution was cooled to 0–5 °C in an ice bath and treated dropwise with 15.0 mL (0.116 mol) of isobutyl chloroformate. After 1.5 h at 0–5 °C, the mixture was treated with a mixture of 28.0 g (0.116 mol) of diethyl L-glutamate hydrochloride, 15.8 mL (0.116 mol) of triethylamine, and 100 mL of dry DMF. The mixture was stirred for 24 h at ambient temperature. Solvent was removed under reduced pressure, and the residue was stirred with 400 mL of 5% $NaHCO_3$ and 400 mL of ether for 1 h. After filtration, the cake was washed with H_2O and dried to leave 26.0 g of the diester (**6a**).

The diester was dissolved in 200 mL of 2-methoxyethanol and treated with 100 mL of 1 N NaOH. The solution was kept at room temperature for 4 h and then diluted with 1 L of H_2O. The solution was acidified with HOAc until precipitation was complete. After refrigeration for 24 h, the product was collected, washed with H_2O, and dried to leave 15.5 g (61%) of crude 10-DA, which assayed at 91% purity by HPLC. Ultimate purification was achieved by chromatography on 300 g of C_{18} reverse-phase adsorbent (Waters Bondapak)[12] in a low-pressure apparatus: UV max at pH 13 254 nm (ϵ 33 170), 375 (7460); NMR Me_2SO-d_6) δ 2.05 (2 H, m, CH_2), 2.3 (2 H, m, CH_2COOH), 3.18 (4 H, s, $CH_2CH_2C_6H_4$), 4.4 (1 H, m, NHCH), 6.75 (2 H, s, NH_2), 7.35 and 7.82 (4H, doublets, C_6H_4), 7.7 (2 H, s, NH_2), 8.50 (1 H, d, CONH), 8.59 (1 H, s, C-7 H); HPLC showed a purity of 99%. The UV, NMR, and HPLC were in agreement with material prepared by the unequivocal syntheses.[1,6]

The 10-alkyl analogues (**7b–d**) were prepared by a similar procedure. The diethyl ester intermediates were chromatographed on silica gel with elution by 5–10% CH_3OH in $CHCl_3$,[13] followed by saponification as above to afford the target acids in greater than 98% purity by HPLC analysis. However, analytical samples of the 10-alkyl analogues (**7b–d**) were obtained directly from HPLC purifications.

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