10-Propargylaminopterin and Alkyl Homologues of Methotrexate as Inhibitors of Folate Metabolism

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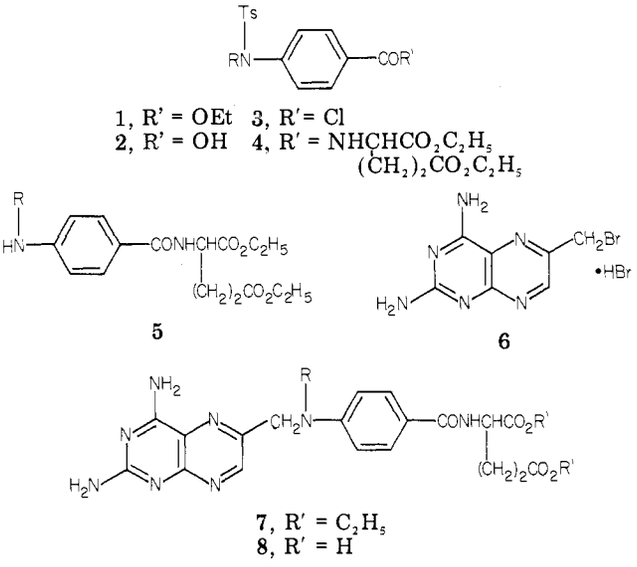
Reported antifolate activity against leukemia L1210 by *N*-[4-[[(2-amino-4-hydroxy-6-quinazolinyl)methyl]-propargylamino]benzoyl]-L-glutamic acid through potent inhibition of thymidylate synthase (EC 2.1.1.45) prompted us to include the propargyl group in a study of the effect on folate metabolism and membrane transport of replacing the 10-methyl group of methotrexate with other groups. Along with the propyl (**8a**) and octyl (**8b**) homologues of methotrexate, the propargyl compound **8c** was prepared for evaluation. Syntheses of **8a,b** were achieved by a standard multistep sequence involving preparation of the side-chain precursors via tosylated intermediates and then their alkylation with 6-(bromomethyl)-2,4-pteridinediamine hydrobromide. The side-chain precursor to **8c** was prepared by direct alkylation of diethyl *N*-(4-aminobenzoyl)-L-glutamate with propargyl bromide and was separated from unchanged amine and dipropargyl coproduct by a combination of methods, including dry-column chromatography and recrystallization. Subsequent steps leading to **8c** were like those used to prepare **8a,b**. Biological evaluations of the three compounds consisted of studies of their effects on enzyme inhibition [(dihydrofolate reductase (EC 1.5.1.3) and thymidylate synthase)], L1210 cell growth inhibition, cellular membrane transport with various murine cell types (L1210, S180, Ehrlich, and epithelial), in vivo (mice) activity vs. L1210 leukemia and S180 ascites, and plasma clearance in mice. The in vivo results vs. S180 ascites offered evidence that **8c** might have a better therapeutic index against this tumor than methotrexate, but no other result from either of these compounds suggested significant superiority over methotrexate.

Recently, *N*-[4-[[(2-amino-4-hydroxy-6-quinazolinyl)-methyl]propargylamino]benzoyl]-L-glutamic acid, which may be viewed as the 10-propargyl derivative of the quinazoline analogue of folic acid, was reported to be a potent antifolate inhibitor of thymidylate synthase (TS, EC 2.1.1.45), inhibiting the enzyme from L1210 cells competitively with respect to the substrate methylenetetrahydrofolate.[1] The reported findings prompted us to prepare the aminopterin analogue bearing the propargyl group at the 10-position (**8c** as shown in Scheme I).[2] In this report we describe the synthesis and results from biological evaluation of **8c** and also those of the propyl (**8a**) and octyl (**8b**) analogues which were prepared as part of an investigation of structural specificities for binding to dihydrofolate reductase (DHFR, EC 1.5.1.3) and to study the effects of such structural changes on folate transporting systems.[3,4]

Chemistry. The diethyl esters **7a–c** from which target compounds **8a–c** were obtained were prepared as outlined in Scheme I from the appropriate side-chain precursors **5a–c** and 6-(bromomethyl)-2,4-pteridinediamine hydrobromide (**6**). Syntheses of **5a,b** were achieved through adaptations of a reported synthesis of diethyl *N*-[4-(methylamino)benzoyl]-L-glutamate.[5] The sequence consisted of NaH-promoted alkylation of ethyl 4-[[(4-methylphenyl)sulfonyl]amino]benzoate to give **1a,b** followed by hydrolysis of the ester groups. The resulting acids **2a,b** were converted to the aroyl chlorides **3a,b**, which were coupled with diethyl L-glutamate to provide **4a,b**; finally, removal of the (4-methylphenyl)sulfonyl (tosyl) groups by treatment with HBr in acetic acid containing phenol gave **5a,b**. This synthetic route is not applicable to **5c** because of the incompatibility of the propargyl group with the conditions for tosyl removal. The approach used to prepare **5c** was direct alkylation of diethyl *N*-(4-aminobenzoyl)-L-glutamate with propargyl bromide. The reaction conditions used were adapted from a reported preparation of ethyl 4-(propargylamino)benzoate[6] and produced a mixture of starting amine, desired product, and dipropargyl coproduct. Unchanged amine was removed by extracting it from the less basic propargylated products with 1 N HCl, and **5c** was then separated from the dipropargyl coproduct by use of dry-column chromatography followed by recrystallization.

Scheme I[a]



[a] a series, R = $CH_3(CH_2)_2$; b series, R = $CH_3(CH_2)_7$; c series, R = $HC{\equiv}CCH_2$.

Biological Evaluation.[7,8] In an earlier paper, we re-

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(1) Jones, T. R.; Calvert, A. H.; Jackman, A. L.; Brown, S. J.; Jones, M.; Harrap, K. R. *Eur. J. Cancer* **1981**, *17*, 11.
(2) We learned while this paper was being written that M. G. Nair, University of South Alabama, Mobile, AL, also recently prepared **8c** by the method described herein.
(3) Sirotnak, F. M.; Chello, P. L.; Moccio, D. M.; Piper, J. R.; Montgomery, J. A.; Parham, J. C. *Biochem. Pharmacol.* **1980**, *29*, 3293.
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(5) Santi, D. V. *J. Heterocycl. Chem.* **1967**, *4*, 475.
(6) Wolf, V. *Leibigs Ann. Chem.* **1952**, *576*, 35.
(7) General methodology used in the evaluation of potential antifolates with regard to transport and pharmacokinetic properties was recently reviewed by Sirotnak, F. M. *Pharmacol. Ther.* **1980**, *8*, 71.

0022-2623/82/1825-0877$01.25/0

Table I. Summary of Biochemical and Growth Studies with Rodent Neoplastic Cells in Vitro[a]

compd	IC_{50}, nM (L1210 cells)	L1210 DHFR inhibn: K_i, nM	membrane transport: influx: K_m (K_i), μM epithelial	S180	Ehrlich	L1210	efflux: k, min^{-1} L1210
8a	2.2	0.003	422	3.5	4.2	3.2	0.26
8b	217	17.5	1294	1.8	1.3	5.6	0.89
8c	1.4	0.5	385	2.1	2.4	3.2	0.26
aminopterin	1.2	0.004			2.1	1.3	0.21
MTX	2.7	0.006	339	11.4	10.1	3.7	0.23

[a] The methods used in these studies have been described; see ref 3 and 9.

Table II. Summary of Data For S180 Ascites Tumor

compd	Rx (schedule)	Rx,[b] mg/kg	mice (no. × expts)	wt. at term., g ± SE	MST, days ± SE	ILS, %
	e2d × 5sc		5 × 2	25.5 ± 4.9	11.6 ± 1.2	
MTX		15	5 × 2	24.4 ± 3.1	21.4 ± 3.6	82
8c		288	3 × 2	22.4 ± 5.5	19.8 ± 5.9	67
	e1d × 5sc		5 × 2	27.5 ± 4.9	10.0 ± 1.2	
MTX		6	5 × 2	24.1 ± 5.6	17.8 ± 1.6	78
8c		36	3 × 2	23.9 ± 4.0	22.9 ± 5.1	129

[a] Methods are described in ref 9. [b] Approximate LD_{10} dosage for MTX on both schedules and for **8c** on a schedule of e1d × 5.

Table III. Summary of Therapy Data For L1210 Leukemia[a]

compd	Rx,[b] mg/kg	mice (no. × expts)	wt at term., g ± SE	MST, days ± SE	ILS, %
control		5 × 12	20.7 ± 1.6	6.8 ± 0.6	
MTX	15	5 × 12	19.8 ± 3.2	17.1 ± 1.9	+152
8a	288	3 × 2	21.3 ± 4.6	15.8 ± 2.4	133
8b	144	3 × 2	22.7 ± 0.1	8.1 ± 0.3	16
8c	288	3 × 3	22.3 ± 0.6	16.6 ± 3.1	143

[a] Methods used are described in ref 10. [b] e2d × 5 of approximate LD_{10} sc dosage for MTX.

Table IV. Plasma Clearance of Folate Analogues in Mice[a]

compd[b]	plasma level,[a] mg/mL × 10^2: 0.2 h	1 h	2 h	4h	7 h	16 h	24 h
MTX	76	8.2	0.93	0.31	0.19	0.09	0.06
8a	54	5.1	0.37	0.16	0.13	0.05	0.03
8b	82	6.4	0.45	0.20	0.16	0.08	0.05
8c	68	3.2	0.12	0.05	0.03	0.01	

[a] Methods described in ref 10 and 11. [b] n = 2-4 mice per point (av ± 18% SE). [c] Dosage = 12 mg/kg sc.

ported data from membrane transport studies in L1210 cells showing that introduction of aralkyl substituents as well as small alkyl groupings at the 10-position of aminopterin results in only small reductions in influx.[3] The transport data for the propyl compound **8a**, the propargyl compound **8c** and methotrexate (MTX) listed in Table I are in keeping with those earlier results in that K_m (K_i) values are increased by only 2- to 3-fold compared with aminopterin. (Test results from **8a** included in the earlier study are repeated here for comparison with **8c**.) The octyl compound **8b** displayed a somewhat greater reduction in influx. The efflux of the compounds studied earlier bearing bulky aralkyl groupings was about the same as aminopterin, but **8b** showed a distinct increase.

Results from the L1210 DHFR inhibition and L1210 cell growth inhibition studies (also listed in Table I) appear to correlate with respect to one another. Tighter binding to DHFR by **8a** and **8c** compared with **8b** is reflected in more pronounced inhibition of cell growth.

All three of the new analogues (**8a–c**) are more effectively transported into S180 and Ehrlich cells (Table I) than MTX but are less effectively transported in epithelial cells of the gut—in the case of the octyl compound **8b** only about one-fourth as well. Unfortunately, the more rapid efflux of **8b** from L1210 cells, and probably other tumor cells, could offset any potential therapeutic advantage. Preliminary evidence, however, suggests that **8c** may have a better therapeutic index than MTX against S180 (Table II). On the schedule employed, **8a** and **8c** appear to be as effective against L1210 in mice as MTX but at much higher dose levels (Table III). The octyl compound, on the other hand, is ineffective.

Although, as mentioned above, all three of these analogues showed somewhat lower transport into gut epithelial cells than MTX, the extremely low toxicity of these compounds in mice was unexpected. This can be partially explained by their more rapid plasma clearance (Table IV). In the case of **8b**, ineffective binding to DHFR and more rapid efflux in normal tissue might be anticipated from the L1210 data, which also accounts for its lack of activity against L1210 cells in vitro and in vivo.

Table V. Summary of Bacterial Studies

compd	IC_{50}, ng/mL: *S. faecium*[a] ATCC 8043	/MTX	*L. casei*[a] ATCC 7469	/MTX	IC_{50}, μM: *L. casei* enzyme[b] DHFR	TS
8b	0.41	670	0.017	>10 000	0.012	>80
8c	0.21	>10 000	0.014	>10 000	0.010	20
MTX	0.09	7 200	0.013	>10 000	0.012	75

[a] Methods described in ref 12. [b] See ref 13.

Table VI

no.	yield, %	mp, °C	mol formula
1a	62	oil[a]	$C_{19}H_{23}NO_4S$
2a	92	147	$C_{17}H_{19}NO_4S$[b]
3a	100	oil[a]	$C_{17}H_{18}ClNO_3S$
4a	81	oil[a]	$C_{26}H_{34}N_2O_7S$
5a	61	65-67	$C_{19}H_{28}N_2O_5$[b]

[a] Used directly in the succeeding step. [b] Anal. C, H, N.

Table VII

no.	yield, %	mp, °C	mol formula
1b	81	oil[a]	$C_{24}H_{33}NO_4S$
2b	61	178-179	$C_{22}H_{29}NO_4S$[b]
3b	100	oil[a]	$C_{22}H_{28}ClNO_3S$
4b	82	68-70	$C_{31}H_{44}N_2O_7S$[b]
5b	43	69	$C_{24}H_{38}N_2O_5$[c]

[a] Used directly in the succeeding step. [b] Anal. C, H, N. [c] Anal. C, H.

The bacterial data (Table V) contrasts with the leukemia data in that there is little difference in the inhibition of DHFR from *Lactobacillus casei* or in the IC_{50} values for the growth of this bacterium. In the case of *Streptococcus faecium*, the IC_{50} of **8c** is about twice that of MTX and one-half that of **8b**, but both compounds are effective. As might be expected, little activity was seen against the bacteria resistant to MTX, although the activity of **8b** against the MTX-resistant *S. faecium* is of some interest. Since compound **8c** was synthesized in the hope that it might be an effective inhibitor of both DHFR and TS, the relatively weak inhibition of TS exerted by **8c** was disappointing, although it is about four times as effective as MTX. The differential in IC_{50} values for DHFR and TS (2000-fold) would indicate that inhibition of the latter enzyme plays little part in the growth-inhibiting properties of **8c**.

Experimental Section

^{1}H NMR (determined in Me_2SO-d_6 with a Varian XL-100-15 spectrometer) and UV spectra (determined in 0.1 N HCl, pH 7 buffer, and 0.1 N NaOH with a Cary 17 spectrophotometer) obtained for final products **8a–c** were consistent with assigned structures. Analytical results indicated by element symbols were within ±0.4% of the theoretical values. Spectral determinations and some of the elemental analyses were performed in the Molecular Spectroscopy Section of Southern Research Institute under the direction of Dr. W. C. Coburn, Jr. Elemental analyses were also performed by Galbraith Laboratories, Knoxville, TN. Examinations of intermediates (including esters of final products) by TLC were performed on Analtech precoated (250 μm) silica gel G(F) plates. Examinations of final products by TLC were performed on Analtech Avicel F plates developed with standard pH 7 buffer solution (Sargent-Welch). Assays by high-performance liquid chromatography (HPLC) were done with equipment from Waters Associates (Model 6000A pump, U6K injector, differential UV detector monitored at 254 nm, 30 × 0.39 cm C_{18} μBondapak column) using a reverse-phase system with a mobile phase of 15% MeCN in 0.1 M NaOAc, pH 3.6. Evaporations were performed with a rotary evaporator and a H_2O aspirator, and products were dried in vacuo (<1 mm) at room temperature over P_2O_5 and NaOH pellets. Final products **8a–c** were dried and then allowed to equilibrate with ambient conditions of the laboratory.

Diethyl *N*-[4-(Propylamino)benzoyl]-L-glutamate (5a). The five-step sequence via intermediates **1a–4a** beginning with NaH-promoted alkylation of ethyl 4-[[(4-methylphenyl)-sulfonyl]amino]benzoate was carried out as described for the synthesis of the Me homologue.[5] Results are listed in Table VI. 4-[[(4-Methylphenyl)sulfonyl]propylamino]benzoic acid (**2a**) was obtained crystalline, but **1a**, **3a**, and **4a** were clear oils. Detosylation of **4a** led to **5a** as a white crystalline residue following evaporation of the CH_2Cl_2 solution used in the direct adaptation of the reported isolation procedure. Further purification was not necessary; the sample of **5a** thus obtained was homogeneous by TLC ($CHCl_3$–MeOH, 95:5).

Diethyl *N*-[4-(octylamino)benzoyl]-L-glutamate (5b) was obtained via intermediates **1b** through **4b** as indicated above for **5a**. Results are listed in Table VII. Following treatment of **4b** with 30% dry HBr–AcOH solution containing C_6H_5OH, the reaction solution was combined with Et_2O, but no precipitate formed. Addition of hexane, however, caused separation of a yellow oil. Decantation of the supernatant followed; then treatment of the oil with petroleum ether (bp 38–55 °C) caused solidification. Subsequent purification steps were like those reported for the Et_2O-precipitated Me homologue,[5] except **5b** was recrystallized from EtOH–H_2O.

Diethyl *N*-[4-(Propargylamino)benzoyl]-L-glutamate (5c). A mixture of diethyl *N*-(4-aminobenzoyl)-L-glutamate (8.06 g, 25.0 mmol), propargyl bromide (3.80 g as 80% solution in toluene, 25.5 mmol), $NaHCO_3$ (2.66 g, 31.7 mmol), and anhydrous EtOH (50 mL) was refluxed with stirring for 16 h. Examination by TLC (EtOAc–cyclohexane, 1:1) revealed unchanged amine, desired product, and a coproduct presumed to be the dipropargyl compound; R_f values 0.23, 0.47, and 0.60, respectively. The dark mixture was filtered, and the filtrate was added dropwise to stirred 1 N HCl (100 mL) to cause precipitation of a dark oil. The supernatant was largely removed by decantation, and the oil was then stirred with 1 N HCl (25 mL) for about 10 min. Supernatant was again decanted off, and the residue, now semisolid and free of starting amine, was dried in vacuo. This crude product mixture was dissolved in the minimum of EtOAc, and the solution was applied to a dry silica gel column (Silica Woelm TSC), which was developed with EtOAc-cyclohexane (1:1). Dark impurities remained near the top of the column. Examination by TLC at intervals along the column revealed an intermediate region enriched in desired product but not free of the presumed dipropargyl coproduct. This section was removed and extracted with EtOH. Evaporation gave a pale-yellow, viscous oil (4.5 g). A reprecipitation from EtOAc (20 mL) by addition of cyclohexane (200 mL) gave a crude solid, which was dissolved in warm 2-PrOH (25 mL). This solution was kept in a refrigerator for about a week while pure crystalline **5c** gradually separated; yield 22% (2.03 g); mp 98–100 °C (lit.[1] mp 98–99 °C).

Diethyl *N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]-propylamino]benzoyl-L-glutamate (7a). A stirred mixture of **6**·0.5*i*-C_3H_7OH (2.49 g, 6.80 mmol) and **5a** (2.70 g, 7.42 mmol) in Me_2NAc (30 mL) was kept at 50–55 °C for 4 h; solution occurred after 1 h. Dropwise addition of the cooled solution to rapidly stirred H_2O (150 mL) containing Et_3N (15 mmol) gave a yellow solid (3.8 g), which was recrystallized from EtOH to give pure **7a**·H_2O: mp 192 °C and homogeneous by TLC ($CHCl_3$–MeOH, 9:1); yield 54% (2.05 g). Anal. ($C_{26}H_{34}N_8O_5$·H_2O)C, H, N.

Diethyl *N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]octyl-amino]benzoyl]-L-glutamate (7b). A mixture of **6**·0.5*i*-C_3H_7OH (3.02 g, 8.25 mmol) and **5b** (4.0 g, 9.20 mmol) with Me_2NAc (50 mL) was stirred at 50–55 °C for 6 h; solution occurred after 5 h. The solution was left at 25 °C for 18 h and then treated with $(i\text{-Pr})_2NEt$ (2.38 g, 18.5 mmol). This solution was combined with H_2O (100 mL) to give crude **7b** as a light-brown solid. Recrystallization from EtOH followed, and pure **7b**, mp 145 °C and homogeneous by TLC ($CHCl_3$–MeOH, 9:1), was obtained in 67% yield (3.38 g) as a yellow crystalline solid. Anal. ($C_{31}H_{44}N_8O_5$)C, H, N.

Diethyl *N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]-propargylamino]benzoyl]-L-glutamate (7c). A mixture of **6**·0.5*i*-C_3H_7OH (330 mg, 0.90 mmol), **5c** (400 mg, 1.11 mmol), and

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Me_2NAc (4 mL) was stirred at 25 °C for 18 h, then at 60 °C for 6 h, and finally at 80 °C for 1 h; solution occurred during the last period. The cooled solution was added dropwise to stirred EtOAc (50 mL), and the yellow solid that formed was collected by filtration. The dried solid (wt 0.57 g) was stirred with EtOH–H_2O (25 mL of each), and the solution that formed was treated with 0.3 N NH_4OH until neutral. The yellow precipitate that formed (wt 0.29 g) gave a thin-layer chromatogram ($CHCl_3$–MeOH, 3:1) that revealed the major component as a UV-absorbing spot of approximate R_f 0.87 and also showed at least two minor components; one that gave a pale fluorescent spot of R_f 0.68 and the other a weak UV-absorbing spot of R_f 0.19. The sample (0.29 g) was dissolved in the minimum of MeOH, applied to a 20 × 20 cm Analtech 2-mm thickness silica gel GF plate, and then developed once with $CHCl_3$–MeOH (1:1). The upper band, strongly UV absorbing, was scraped from the plate and stirred with EtOH (200 mL) at 25 °C for 18 h. Evaporation of the clarified EtOH solution gave pure **7c**·H_2O, mp 165–168 °C, as a yellow solid homogeneous according to TLC ($CHCl_3$–MeOH, 1:1 or 3:1): yield 52% (0.26 g). Anal. ($C_{26}H_{30}N_8O_5·H_2O$) C, H, N. Its IR spectrum (KBr) revealed the acetylenic absorption band at 2140 cm^{-1}. In another run, instead of using preparative TLC, the product was purified by a reprecipitation from MeOH–H_2O, followed by two recrystallizations from MeOH: yield 32% (1.05 g from 6.20 mmol of **6** and 6.90 mmol of **5a**).

***N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]propylamino]benzoyl]-L-glutamic Acid (8a).** A solution of **7a**·H_2O (900 mg, 1.63 mmol) in EtOH (170 mL) at 35 °C was treated with 1 N NaOH (4.2 mL), and the solution was kept at 35 °C for 4.5 h and then left at 25 °C for 16 h. The mixture, now containing yellow solid, was treated with H_2O (10 mL), and the solution was evaporated (H_2O aspirator, bath to 30 °C) to remove EtOH. The residue was dissolved in H_2O (50 mL), and the solution was treated with 1 N HCl to pH 3.7 to precipitate **8a**, which was collected after 2 h in a refrigerator: yield 89% (750 mg); homogeneous by HPLC. Anal. ($C_{22}H_{26}N_8O_5·1.8H_2O$) C, H, N.

***N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]octylamino]benzoyl]-L-glutamic Acid (8b).** The ester **7b** (3.36 g, 5.51 mmol) was converted to **8b** as described for the preparation of **8a**. The yield of pure **8b**·H_2O, homogeneous by TLC, was 89% (2.8 g). Anal. ($C_{27}H_{36}N_8O_5·H_2O$)C, H, N.

***N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]propargylamino]benzoyl]-L-glutamic Acid (8c).** A solution of **7c** (1.05 g, 1.96 mmol) in MeOH (115 mL) was treated with 1 N NaOH (4.7 mL), and the solution was left at 25 °C for 18 h and then boiled under reflux for 1 h. Evaporation under reduced pressure (H_2O aspirator, bath 20–25 °C) followed, and the residue was dissolved in H_2O (40 mL). The solution was treated with Norit, filtered (Celite mat), and acidified to pH 3.6 by dropwise treatment with 1 N HCl with stirring. The mixture was kept in an ice–H_2O bath for 2 h before the yellow solid was collected: yield 81% (0.82 g). Anal. ($C_{22}H_{22}N_8O_5·2H_2O$) C, H, N. Assay by TLC and HPLC showed the sample to be homogeneous: ^1H NMR (Me_2SO-d_6) δ 2.0 (m, $CHCH_2CH_2$), 2.3 (m, CH_2CO_2H), 3.20 (s, $CH_2C{\equiv}CH$), 4.4 (m, $NHCHCO_2H$), 4.47 (s, $CH_2C{\equiv}CH$), 4.82 (s, CH_2N), 6.7 (s, NH_2), 6.9 and 7.8 (2d, C_6H_4), 7.6 (br s, NH_2), 8.2 (d, CONH), 8.69 (s, C_7 H).

Under the HPLC conditions used, **8c** gave a peak with time of retention near 7.7 min, while folic acid and aminopterin had retention times near 3.5 and 3.8 min, respectively. In a deliberate mixture under these conditions, folic acid and aminopterin are barely resolved from one another, but each is distinctly resolved from **8c**. Hydrolysis of pure **7c** as described was carried out three times with the same results; assay of each of these samples by HPLC showed **8c** thus prepared to be homogeneous.

Acknowledgment. This investigation was supported by the National Cancer Institute through the following CA grants: 25236 (work at Southern Research Institute); 10914 (work at Tufts University); 08748, 18856, and 22764 (work at Memorial Sloan-Kettering Cancer Center). Support for the work at Memorial Sloan-Kettering Cancer Center was also provided by a grant from the Pardee Foundation.