Folate Analogues. 26. Syntheses and Antifolate Activity of 10-Substituted Derivatives of 5,8-Dideazafolic Acid and of the Poly-γ-glutamyl Metabolites of N^{10}-Propargyl-5,8-dideazafolic Acid (PDDF)[1]

M. G. Nair,*† Nitin T. Nanavati,† Indira G. Nair,† Roy L. Kisliuk,‡ Y. Gaumont,‡ M. C. Hsiao,§ and Thomas I. Kalman§

Department of Biochemistry, University of South Alabama, College of Medicine, Mobile, Alabama 36688, Department of Biochemistry and Pharmacology, Tufts University, School of Medicine, Boston, Massachusetts 02111, and Departments of Medicinal Chemistry and Biochemical Pharmacology, State University of New York, Buffalo, New York 14260.
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The poly-γ-glutamyl derivatives of N^{10}-propargyl-5,8-dideazafolic acid (PDDF) with a chain length of up to five glutamate residues were synthesized from N^{10}-propargyl-5,8-dideazapteroic acid (**7**) by the solid-phase procedure. These compounds were evaluated for their antifolate activity using folate-requiring microorganisms and intact and permeabilized L1210 cells and as inhibitors of dihydrofolate reductase and thymidylate synthase derived from *L. casei*. The polyglutamylated derivatives of PDDF (**1**) were more active than the parent compound in inhibiting the growth of *L. casei*, thymidylate synthesis in permeabilized L1210 cells, and *L. casei* thymidylate synthase. Two analogues of 5,8-dideazafolic acid (**2** and **3**), one with a 2-butyne and another with a cyclopropylmethyl substituent at N^{10}, were also synthesized and evaluated for their antifolate activities using the above-mentioned test systems. They were considerably less active than PDDF or its polyglutamylated derivatives. N^{10}-Propargyl-5,8-dideazapteroyl tri-, tetra-, and pentaglutamates were equipotent with 5-fluorodeoxyuridylate as inhibitors of thymidylate synthesis in permeabilized L1210 cells. The polyglutamyl metabolites of PDDF were shown to be the most potent antifolate inhibitors of *L. casei* and L1210 thymidylate synthases yet described.

Classical antifolate drugs such as methotrexate (MTX), 10-deazaaminopterin (10-DAAM),[2,3] and 10-ethyl-10-deazaaminopterin (10-EDAAM)[2,3] are metabolized to their respective poly-γ-glutamyl derivatives in human red cells,[4,5] normal murine tissues,[5,6] and various tumor cells in vivo and in culture.[6-9] Like the parent drugs, these metabolites are also powerful antifolates by virtue of their strong inhibition of dihydrofolate reductase (DHFR).[10,11] The polyglutamyl metabolites of MTX, 10-DAAM, and 10-EDAAM accumulate preferentially[8] in the tumor compared to the normal proliferative tissues of mouse-bearing L1210 leukemia. The enzyme folylpolyglutamate synthetase (FPGS) has a broad spectrum of substrate specificity[12] and converts most of the classical folate analogues to polyglutamyl derivatives of varying glutamate chain length. Recent studies with classical 4-amino antifolates have indicated that the degree of polyglutamylation is influenced by structural changes at the bridge region.[7,8] It has also been shown that the enzyme folylpolyglutamate hydrolase (FPGH; conjugase), which converts the poly-γ-glutamyl metabolites to their monoglutamate, is less active in tumors than in normal proliferative tissues.[13] The tissue-specific differences in FPGH activity may be partly responsible for the preferential accumulation of these metabolites in tumors. Many investigators have also shown that polyglutamyl metabolites of MTX with higher glutamate chain lengths efflux from various cells at a much slower rate than the parent drug.[14,15] This slower efflux rate in effect partly contributes to the longer persistence of the drug in those tissues, which are capable of enhanced polyglutamylation such as the tumor.

Although the polyglutamylation of 4-amino antifolates has been studied extensively in recent years, such studies on 4-oxyantifolates have not yet been conducted in detail.[16] Homofolate exhibited weak substrate activity toward purified hog liver folylpolyglutamate synthetase.[12] The potent antileukemic agent and thymidylate synthase inhibitor N^{10}-propargyl-5,8-dideazafolate (PDDF)[17] has been shown to be a good substrate of FPGS.[18] This observation strongly indicated the possibility that like other classical 4-amino antifolates, PDDF is also metabolized to its poly-γ-glutamyl metabolites in vivo. The metabolism of radiolabeled PDDF to its poly-γ-glutamyl metabolites in normal mouse tissues has been subsequently confirmed in this laboratory.[19] In order to unravel the role of these

† Department of Biochemistry.
‡ Department of Biochemistry and Pharmacology.
§ Departments of Medicinal Chemistry and Biochemical Pharmacology.

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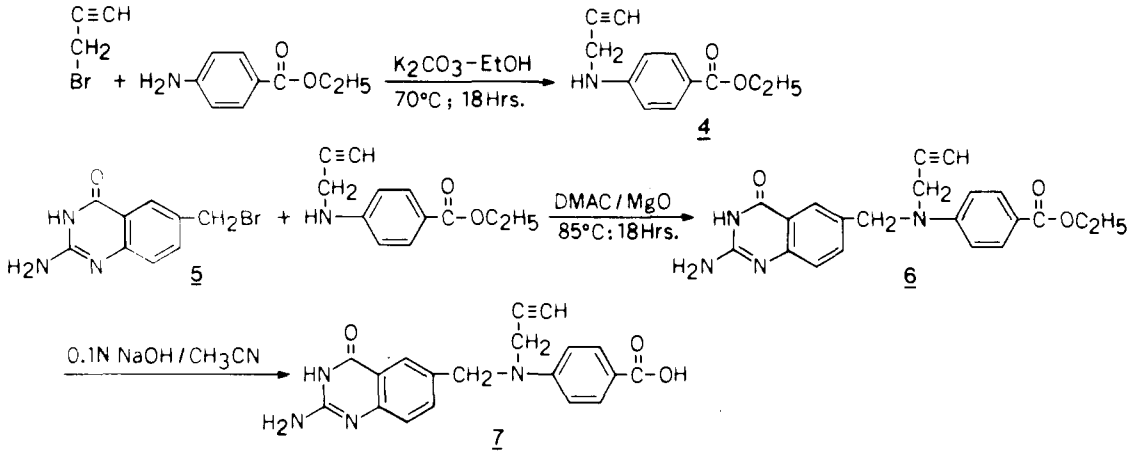
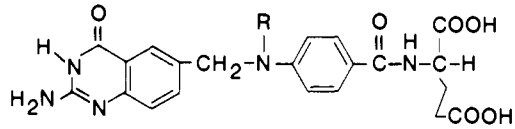
Scheme I



metabolites in the overall chemotherapeutic effectiveness of PDDF, large amounts were needed for various biochemical and pharmacological studies.[11,20] The results of such studies might be useful in the design of better antifolate drugs, taking into account the less exploited phenomenon of their polyglutamylation. As part of a continuing program aimed at the development of more specific and less toxic antifolate drugs, this paper details the chemical synthesis and preliminary biological evaluation of the poly-γ-glutamyl metabolites of PDDF and two N^{10}-substituted analogues (**2** and **3**) of 5,8-dideazafolic acid.



1 R = $-CH_2-C\equiv CH$

2 R = $-CH_2-C\equiv C-CH_3$

3 R = $-CH_2-$ 

Chemistry. The preparation of the poly-γ-glutamyl metabolites of PDDF with varying chain length by the solid-phase procedure was dependent on the availability of the key intermediate, N^{10}-propargyl-5,8-dideazapteroic acid (**7**), which was synthesized according to the following procedure. Ethyl *p*-aminobenzoate was allowed to react with propargyl bromide in ethanol in the presence of K_2CO_3 to give a mixture of the mono- and dialkylation products. The desired monoalkylation product **4** was separated from the mixture by column chromatography on silica gel. Reaction of 6-(bromomethyl)quinazoline **5**[21] with ethyl *N*-propargyl-*p*-aminobenzoate (**4**) in the presence of MgO at elevated temperature gave ethyl N^{10}-propargyl-5,8-dideazapteroate (**6**) in good yield. The ester **6** was hydrolyzed to the pteroic acid analogue **7**, which was purified by ion exchange chromatography on DEAE-cellulose (Scheme I). The polyglutamyl derivatives of PDDF with a chain length of up to five glutamate residues were synthesized by the solid-phase procedure from **7**.

Merrifield chloromethyl peptide resin was treated with the cesium salt of *tert*-Boc-L-glutamic acid α-benzyl ester to obtain the protected amino acid resin ester. Deprotection was carried out with 25% TFA in CH_2Cl_2, and the deprotected resin ester was neutralized with triethylamine in CH_2Cl_2. The second glutamate moiety was introduced by reacting the neutralized resin with a solution of the mixed anhydride derived from *tert*-Boc-L-glutamic acid α-benzyl ester and isobutyl chloroformate. The resin was then acetylated with a mixture of acetic anhydride and triethylamine to block unreacted amino groups from further reaction.

After the acetylation step, the resin ester was deprotected with TFA, neutralized with TEA, and coupled with the mixed anhydride of *tert*-Boc-L-glutamic acid α-benzyl ester to introduce the third glutamate moiety. Each deprotection and coupling step was preceded by an acetylation step.

Once the desired glutamate chain length was reached on the resin, the resin-bound polyglutamate was deprotected and neutralized for coupling with the pteroic acid analogue. Under a set of carefully controlled conditions a pteroic acid analogue could be selectively activated at the carboxyl group with isobutyl chloroformate. By use of this procedure,[5] N^{10}-propargyl-5,8-dideazapteroic acid was activated to **12** with isobutyl chloroformate and reacted with the deprotected resin-bound poly-γ-glutamate of the desired chain length. After the final coupling, the resin-bound product was treated with a 1:1 mixture of 2 N NaOH and *p*-dioxane to cleave the product from the resin and simultaneously remove the benzyl protecting groups. The crude products were purified by ion exchange chromatography on DEAE-cellulose (Scheme II). These compounds eluted serially from the column at NaCl con-

Table I. Ultraviolet Spectra, Chromatographic Properties, and Yield of PDDF Derivatives

compd	UV (0.1 N NaOH) λ_{max}, nm	retention time, min[a]	NaCl,[b] M	yield, %
PDDF (PDDP-G_1)[c]	301.0, 279.2	17.5	0.245	
PDDP-G_2	301.2, 279.5	14.5	0.288	38.8
PDDP-G_3	301.7, 279.1	12.6	0.320	32.0
PDDP-G_4	302.6, 280.1	10.8	0.348	24.0
PDDP-G_5	302.9, 279.6	9.0	0.379	22.6

[a] The retention times of PDDF and its polyglutamyl metabolites were obtained by reverse-phase HPLC on an Applied Science C_{18} μBondapak column. The compounds were injected as their ammonium salt in distilled water and eluted at 1.0 mL/min using a 20-min linear gradient from 5 to 25% acetonitrile in 0.1 M sodium acetate at pH 5.1. The eluent was monitored at 254 nm using a Waters Model 440 absorbance detector. [b] These values are NaCl concentrations (M) required to elute each compound from a DEAE-cellulose column (2.5 × 20 cm) at pH 7.00. [c] PDDP-G_1, N^{10}-propargyl-5,8-dideazapteroyl monoglutamate (PDDF).

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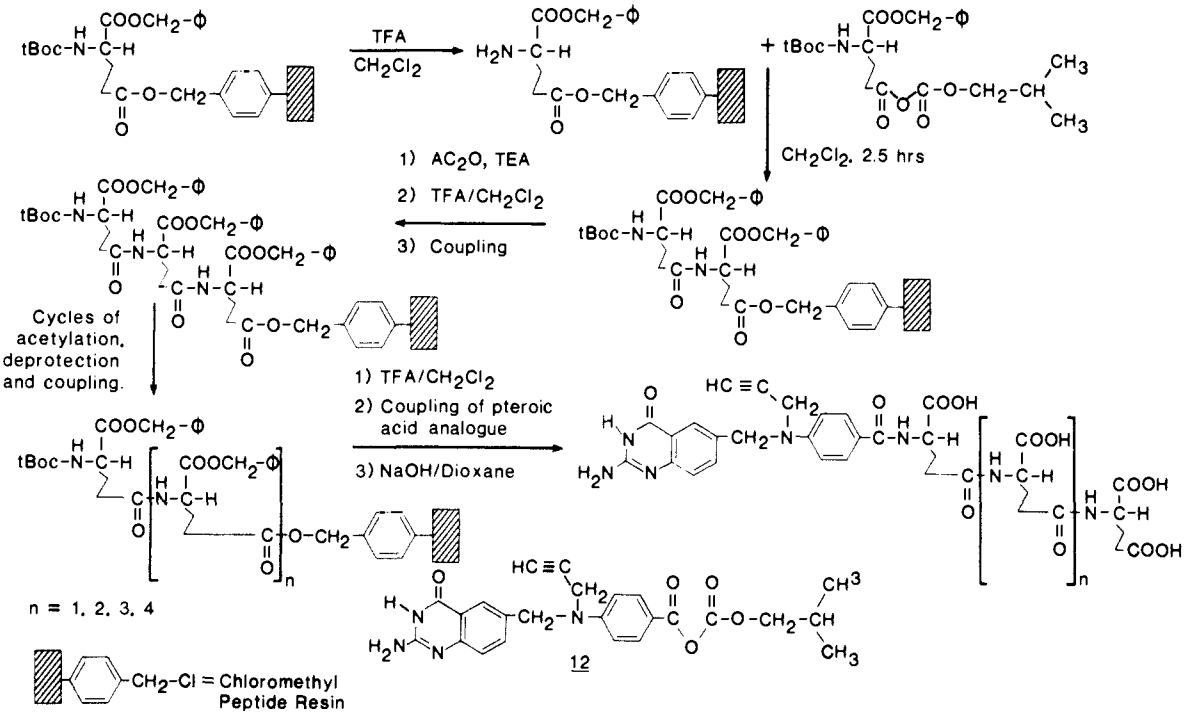
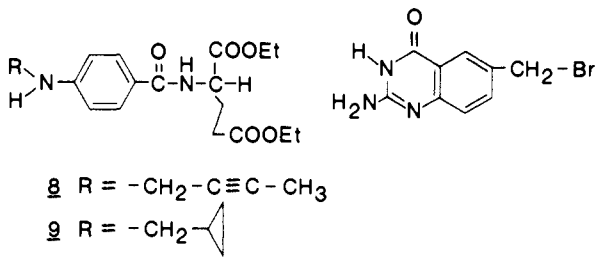
Scheme II



centrations that were dependent on the number of free carboxyl groups on the glutamate chain. They can also be separated by HPLC on a C_{18} μBondapak column (Table I).

We have previously encountered the instability of the *N*-propargyl group of folate analogues toward base. During an attempt to prepare N^{10}-propargylfolic acid from N^{10}-propargylaminopterin by deamination with base, depropargylation took place with formation of folic acid.[22] Since the resin-bound polyglutamate derivatives were cleaved from the resin by using a mixture of 2 N NaOH and dioxane, it was necessary to examine whether depropargylation took place during this final step of the synthesis. Therefore, two polyglutamyl derivatives were examined by NMR for the presence of the propargyl group. These compounds exhibited the bridge methylene and the propargyl methylene proton resonances as two broad singlets around 4.3 and 4.8 ppm. The resonance of the α-proton of the glutamate moiety appeared between these signals around 4.6 ppm. The combined integrated intensities of these three signals were compared with the integrated intensities of the four glutamate protons and the acetylenic proton appearing between 2 and 3 ppm. For PDDF, these integrated intensities were equal, whereas for PDDP-G_2 they were in a 1:2 ratio, and for PDDP-G_3 a 1:3 ratio. These results clearly established that depropargylation did not take place during the cleavage of the products from the resin and provided evidence as to the correctness of the structures of these polyglutamates. All the polyglutamyl derivatives of PDDF were quantitated spectrophotometrically before use and were stored frozen as ammonium salts in distilled water.

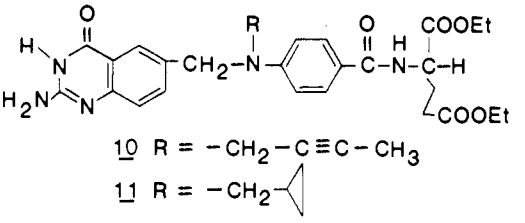
The dideazafolate analogues **2** and **3** were synthesized as follows. The mesylate of 2-butyne-1-ol was prepared by a standard procedure. Reaction of this mesylate with diethyl (*p*-aminobenzoyl)-L-glutamate in the presence of K_2CO_3 gave the monoalkylation product **8**, which was contaminated with the dialkylation product and the unreacted amine. The alkylation product **9** was prepared by reacting (bromomethyl)cyclopropane with diethyl (*p*-aminobenzoyl)-L-glutamate and K_2CO_3 in ethanol. The crude products **8** and **9** of each reaction were purified by column chromatography on a silica gel column. Reaction of **8** or **9** with 6-(bromomethyl)quinazoline **5** in DMAC in the presence of MgO as an acid scavenger at elevated



8 R = $-CH_2-C\equiv C-CH_3$

9 R = $-CH_2-$(cyclopropyl)

temperatures gave the diethyl esters **10** and **11**, respectively. These esters were hydrolyzed with NaOH in



10 R = $-CH_2-C\equiv C-CH_3$

11 R = $-CH_2-$(cyclopropyl)

MeCN to the target compounds **2** and **3**. The NMR spectra of **2** and **3** exhibited characteristic resonances of the 2-butyne substituent and the high-field signals of the cyclopropyl protons, respectively, at the expected frequencies.

Biological Evaluation and Discussion

Successive increments in the polyglutamyl chain length of classical 4-amino antifolates such as MTX and 10-DAAM resulted in decreasing potencies of these metabolites against the growth of *Lactobacillus casei*.[11] However, elongation of the polyglutamyl chain length of MTX did not increase the IC_{50} of these compounds toward *L. casei* dihydrofolate reductase. Therefore, it was concluded that

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Table II. Antimicrobial Activities of PDDF Derivatives and Analogues

compd	IC_{50}, nM			
	Streptococcus faecium		*Lactobacillus casei*	
	ATCC 8043	MTX-resistant	ATCC 7469	MTX-resistant
PDDP-G_1	0.13	20	0.4	6400
PDDP-G_2	0.36	125	0.05	280
PDDP-G_3	34	>800	0.08	1100
PDDP-G_4	75	>800	0.18	>20000
PDDP-G_5	>80	>800	0.35	>20000
N^{10}-(cyclopropylmethyl)-5,8-dideazafolate	2.6	1500	8.1	>20000
N^{10}-(methylpropargyl)-5,8-dideazafolate	6.7	1600	86	>20000
MTX	0.3	3900	0.03	>1000000

Table III. Inhibition of Thymidylate Synthase and Dihydrofolate Reductase by PDDF Derivatives and Analogues

compd	IC_{50}, M			
	dihydrofolate reductase		thymidylate synthase	
	S. faecium	*L. casei*	*S. faecium*	*L. casei*
PDDP-G_1	6.4×10^{-7}	4.9×10^{-6}	1.0×10^{-8}	2.1×10^{-8}
PDDP-G_2	3.4×10^{-7}	3.1×10^{-6}	1.0×10^{-8}	1.4×10^{-8}
PDDP-G_3	3.7×10^{-7}	5.3×10^{-6}	5.5×10^{-9}	1.4×10^{-8}
PDDP-G_4	3.0×10^{-7}	3.7×10^{-6}	4.7×10^{-9}	1.4×10^{-8}
PDDP-G_5	2.9×10^{-7}	2.9×10^{-6}	6.3×10^{-9}	1.3×10^{-8}
N^{10}-(cyclopropylmethyl)-5,8-dideazafolate		2.3×10^{-6}		1.2×10^{-5}
N^{10}-(methylpropargyl)-5,8-dideazafolate		2.5×10^{-4}		4.4×10^{-6}
MTX	2.7×10^{-9}	8.5×10^{-9}	1.2×10^{-4}	1.7×10^{-4}

MTX polyglutamates of higher chain length may not be efficiently transported across the cell wall. In this context, it was of interest to evaluate the polyglutamyl metabolites of PDDF as inhibitors of the growth of folate-requiring microorganisms. The results are summarized in Table II. Contrary to our expectations, all the polyglutamyl derivatives of PDDF were active against the growth of *L. casei*. The diglutamate (PDDP-G_2) was the most effective. Shane and Stokstad[23] have previously shown that in spite of diminished transport, the tri- and tetraglutamates of pteroic acid support full and 70% of full growth, respectively. It is probable that, like the polyglutamates of MTX, 10-DAAM, and folate, polyglutamyl derivatives of PDDF are also transported more poorly than PDDF in *L. casei*. Therefore, it is likely that the polyglutamyl metabolites of PDDF are extremely potent inhibitors of folate metabolism in this organism. The closely related PDDF analogues **2** and **3** are poorer growth inhibitors than any of the PDDF derivatives tested.

PDDF is weakly active against a MTX-resistant strain of *L. casei*, and activity is enhanced by adding one or two Glu residues (Table II). Further addition of Glu residues diminishes activity. PDDP-G_2, the most inhibitory compound, is 20 times more active than PDDF and at least 3500 times more active than MTX. With *S. faecium* polyglutamylation causes a progressive decrease in inhibitory potency in both sensitive and resistant strains. Compounds **2** and **3** showed moderate activity.

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Table IV. Inhibition of Thymidylate Synthesis in L1210 Cells

compd	intact cells,[a] %, at 10 μM	inhibition, %, at 100 μM	permeabilized cells IC_{50}, M
N^{10}-propargyl-5,8-dideazapteroyl monoglutamate (PDDP-G_1)	55	87	3×10^{-8}
N^{10}-propargyl-5,8-dideazapteroyl diglutamate (PDDP-G_2)	0	32	6×10^{-9}
N^{10}-propargyl-5,8-dideazapteroyl triglutamate (PDDP-G_3)	0	8	2×10^{-9}
N^{10}-propargyl-5,8-dideazapteroyl tetraglutamate (PDDP-G_4)	0	3	1.4×10^{-9}
N^{10}-propargyl-5,8-dideazapteroyl pentaglutamate (PDDP-G_5)	0	8	2×10^{-9}
methotrexate	95	98	6×10^{-5}
5-fluorodeoxyuridylate			2×10^{-9}
3			8.2×10^{-5}
2			1.4×10^{-5}

[a] In the intact cell assay, the IC_{50} values for 10-propargyl-5,8-dideazapteroyl monoglutamate, methotrexate, and N^{10}-(cyclopropylmethyl)-5,8-dideazafolate were 8.2×10^{-6} M, 3.2×10^{-7} M, and 3×10^{-4} M, respectively. At a concentration of 3×10^{-4} M, N^{10}-(methylpropargyl)-5,8-dideazafolate did not show any significant inhibition.

The enzyme inhibitory properties of the polyglutamyl derivatives of PDDF and analogues **2** and **3** were next examined (Table III). With *L. casei* enzymes, the inhibition of dihydrofolate reductase was 200–300 times weaker than the inhibition of thymidylate synthase for each compound tested. By measuring the effect of enzyme concentration on the IC_{50} values,[11] it was established that PDDP-G_3 is a stoichiometric inhibitor of *L. casei* thymidylate synthase. Therefore, from the data of Table III it can be concluded that all the compounds from PDDP-G_2 through PDDP-G_5 are stoichiometric inhibitors of this enzyme. Analogues **2** and **3** were much poor inhibitors of *L. casei* thymidylate synthase than was PDDF. Similar studies with enzymes derived from *S. faecium* show that thymidylate synthase from this organism is also strongly inhibited by PDDF and its polyglutamate forms (Table III). With *S. faecium* enzymes, the inhibition of dihydrofolate reductase is 30–60 times weaker than the inhibition of thymidylate synthase.

It was of interest to investigate the ability of these analogues to block thymidylate synthesis in intact and permeabilized L1210 cells. The permeabilized cellular system permits the evaluation of the effects of PDDF polyglutamates on thymidylate synthase in its natural environment. The results of these studies are summarized in Table IV. In intact cells, the inhibition of thymidylate synthesis was decreased by the addition of γ-glutamyl residues to PDDF. This decreased activity may be accounted for by their poor penetration into L1210 cells. However, in permeabilized cells, the inhibitory activity of PDDF was greatly enhanced by the addition of glutamate residues. Compounds PDDP-G_3 through PDDP-G_5 were equipotent with 5-fluorodeoxyuridylate, a stoichiometric inactivator of thymidylate synthase. In permeabilized L1210 cells, the analogues **2** and **3** were much weaker than all PDDF derivatives in inhibiting thymidylate synthesis. The results obtained with permeabilized L1210 cells are qualitatively similar to those obtained by using *L. casei* thymidylate synthase (Table III).

Analogues **2** and **3** were synthesized to evaluate how reduction of the polarity of the N^{10}-substituent of 5,8-dideazafolate affects the inhibition of thymidylate synthase. The cyclopropylmethyl substituent is relatively nonpolar and contains a three-membered ring in place of the ethynyl group. The 2-butyne substituent is a less polar

homologue of the propargyl group and is similarly symmetrical around the triple bond. Both of these compounds were poor inhibitors of thymidylate synthase. Since the nonpolar N^{10}-ethyl and N^{10}-isopropyl analogues are relatively potent inhibitors,[24] the results suggest that the topography of the active site of the enzyme may impose very strong geometric restrictions toward the N^{10}-substituent, creating favorable binding interactions for the propargyl group, but not for the slightly bulkier substituents of **2** and **3**. These conclusions are in general agreement with the structure–activity correlations recently reported by Jones et al.[24]

A very selective and specific inhibition of thymidylate synthase should result in the blockade of the de novo biosynthesis of dTMP, which is an obligatory intermediate for DNA synthesis. The stoichiometric inhibition of thymidylate synthase exhibited by the polyglutamyl metabolites of PDDF appears to meet this requirement. The enhanced inhibitory activity of the polyglutamyl derivatives of PDDF toward human thymidylate synthase has also been documented.[20] We have recently established the formation of these metabolites in normal mouse tissues.[19] It remains to be established whether, like the 4-amino antifolates, PDDF is preferentially polyglutamylated in the tumor compared to normal proliferative tissues. Further studies are needed to compare the polyglutamylation of PDDF in specific normal proliferative tissues such as the intestinal epithelium and bone marrow with polyglutamylation in tumor cells of tumor-bearing animals. In this way it may be possible to better understand the role of these metabolites in the chemotherapeutic effectiveness of PDDF.

Experimental Section

All organic solvents were dried over a Type-3A molecular sieve before use. Isobutyl chloroformate was freshly distilled and stored over $CaCO_3$. Triethylamine and 4-methylmorpholine were purchased from Aldrich and redistilled prior to use. Ultraviolet spectra were run on a Bausch and Lomb Spectronic Model 2000 spectrophotometer interfaced with a Commodore Superpet computer. NMR spectra were run on a Perkin-Elmer Model R-32 spectrometer. Field strength of proton resonances are expressed in parts per million, and peak multiplicity is depicted as s, singlet; d, doublet; t, triplet; br, broadened singlet; and *c*, unresolved multiplet the center of which is given. Me_4Si was used as an external standard for spectra that were run in TFA as a solvent, unless otherwise specified. Melting points were determined on a Fisher Model 355 digital melting point analyzer. Ion exchange chromatography was carried out by using DEAE-cellulose in the chloride form. The compounds were eluted from the column with a linear NaCl gradient from 0 to 0.5 M, in 0.005 M phosphate buffer at pH 7.0. All HPLC analyses were done on a Waters 6000 A instrument equipped with a Model 660 solvent programmer and U6K injector. Elemental analyses were done by Galbraith Laboratories, Inc., Knoxville, TN. Where analyses were indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.4% of the theoretical values.

N^{10}-Propargyl-5,8-dideazapteroic Acid (7). Compound **4** was prepared according to the procedure recently described from this laboratory.[1] A mixture of 762 mg (3.0 mmol) of 6-(bromomethyl)quinazoline **5**, 609 mg (3.0 mmol) of **4**, and 120 mg (3.0 mmol) of magnesium oxide in 12 mL of DMAC was stirred at 80 °C for 18 h. The clear solution thus obtained was poured over 150 g of crushed ice, stirred, filtered, and dried over P_2O_5 in vacuum: yield 540 mg (48%); mp 255 °C dec; NMR (TFA) δ 7.83, 7.32 (d, *c*, 2 H, 5 H, aromatic), 4.75 (s, 2 H, C^9H_2), 4.2 (br, 2 H, propargyl methylene), 4.1 (q, 2 H, ethoxy), 2.12 (s, 1 H, acetylenic), 1.02 (t, 3 H, ethoxy). This crude product **6** (376 mg, 1 mmol) was stirred at 25 °C with 100 mL (10 mmol) of 0.1 N NaOH for 30 min. To this suspension was added portionwise 50 mL of MeCN, whereupon a clear solution was obtained. After 18 h of stirring, the MeCN was removed by rotary evaporation. The pH of the hydrolysis mixture was adjusted with 1 N HCl to 7.0, and the solution was concentrated to ~50 mL under reduced pressure. The solution was cooled to 4 °C and the pH adjusted to 3.5 with glacial HOAc. The slightly yellow precipitate thus obtained was filtered, washed with distilled water, and dried at 100 °C over P_2O_5 in vacuum: yield 96%. The compound was further purified by ion exchange chromatography over DEAE-cellulose using a linear NaCl gradient from 0 to 0.5 M at pH 7.0: UV (0.1 N NaOH) λ_{max} 278 nm; NMR (TFA) δ 7.8, 7.3, 7.0 (c, 7 H, aromatic), 4.75, 4.25 (s, 4 H, methylenes), 2.36 (s, 1 H, acetylenic). Anal. ($C_{19}H_{16}N_4O_3$) C, H, N.

Diethyl *N*-[4-[(2-Butynyl)amino]benzoyl]-L-glutamate (8). A solution of 2.8 g of 2-butyne-1-ol (40 mmol) and 5.56 mL (49 mmol) of triethyl amine in 100 mL of dry ether was placed in a round-bottom flask and stirred at 0 °C for 15 min. From a dropping funnel 3.2 mL of methanesulfonyl chloride dissolved in 10 mL of ether was added to this solution during 10 min. After the addition was complete, the reaction was allowed to proceed at 0 °C for 20 min and was filtered. The precipitate was washed with 20 mL of ether and the combined filtrate evaporated to dryness at reduced pressure. The NMR spectrum of the viscous oil thus obtained exhibited resonances expected of the mesylate. The mesylate was mixed with 12 g (37.3 mmol) of diethyl (*p*-aminobenzoyl)-L-glutamate and dissolved in 150 mL of chloroform. To this clear solution 4.5 g of powdered K_2CO_3 was added, and the mixture was refluxed for 18 h, concentrated to 50 mL by rotary evaporation, and filtered. The filtrate was chromatographed on a silica gel column by elution with chloroform. The fractions were monitored by TLC, and those corresponding to the product were pooled and evaporated to obtain a crystalline residue: yield 6.363 g (45.6%); mp 81–82 °C; NMR ($CDCl_3$) δ 7.75, 6.66 (d, 4 H, aromatic), 6.75 (t, 1 H, amide), 4.85 (t, 1 H, α-proton of gutamate), 4.2 (c, 4 H, ethoxy), 3.94 (s, propargyl CH_2), 2.5–2.1 (c, 4 H, glutamate), 1.8 (s, 3 H, butyne methyl), 1.25 (*c*, 6 H, ethoxy). Anal. ($C_{20}H_{26}N_2O_5$) C, H, N.

Diethyl *N*-[4-[(Cyclopropylmethyl)amino]benzoyl]-L-glutamate (9). A mixture of 5 g (15.5 mmol) of diethyl (*p*-aminobenzoyl)-L-glutamate and 2.16 g (15.6 mmol) of powdered K_2CO_3 in 100 mL of reagent grade alcohol was stirred in a 500-mL round-bottom flask at 70 °C for 5 min. To this stirring suspension was slowly added 5 g (37 mmol) of bromomethylcyclopropane dissolved in 20 mL of alcohol during a period of 1.5 h. After 27 h, TLC indicated the formation of a major product in ~40% yield. At this stage the reaction mixture was evaporated and the residue triturated with 50 mL of EtOAc. The EtOAc layer was washed 3 times with 50-mL portions of distilled water, dried over anhydrous Na_2SO_4, and evaporated. The resulting glassy residue was dissolved in 25 mL of CH_2Cl_2 and chromatographed on a silica gel column. Elution with CH_2Cl_2 gave the least polar dialkylation product first, which was followed by the monoalkylation product: yield 1.95 g; mp 76–77 °C; NMR ($CDCl_3$) δ 7.7, 6.7 (d, 4 H, aromatic), 4.8 (t, 1 H, α-proton of glutamate), 4.2 (c, 4 H, ethoxy), 3.02 (d, 2 H, methyl cyclopropyl), 2.1–2.6 (c, 4 H, glutamate), 1.3 (c, 6 H, ethoxy), 0.6 (c, 2 H, cyclopropyl), 0.28 (c, 2 H, cyclopropyl). After this work was completed Jones and co-workers[24] published another method for the preparation of **9**. Their melting point and NMR data are slightly different from those of ours. Anal. ($C_{20}H_{28}N_2O_5$) C, H, N.

N^{10}-(2-Butynyl)-5,8-dideazafolic Acid (2). A mixture of 508 mg (2 mmol) of 2-amino-6-(bromomethyl)-4-hydroxyquinazoline (**5**), 758 mg (2 mmol) of alkylation product **8**, and 80 mg (2 mmol) of MgO in 10 mL of DMAc was stirred at 100–105 °C for 18 h. The clear solution thus obtained was poured over 100 g of crushed ice, triturated, filtered, and dried over P_2O_5 in vacuum: yield 1.05 g; mp 216 °C dec. In an Erlenmeyer flask 801 mg (1.5 mmol) of the crude diethyl ester **10** was suspended in 150 mL of 0.1 N NaOH and vigorously stirred under nitrogen. To this suspension was added portionwise 30 mL of MeCN during 20 min. The flask was stoppered under nitrogen, and the stirring continued for 6 h. The MeCN was removed in vacuum at 30 °C by rotary evaporation, and the turbid solution was filtered and acidified

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to pH 4.0 with glacial HOAc. The white precipitate thus obtained was collected by filtration, washed several times with water, and dried over P_2O_5 in vacuum: yield 650 mg; mp 225 °C dec; UV (0.1 N NaOH) λ_{max} 278 (ϵ 22 237), 302 (20 534) nm; NMR (TFA) δ 7.79 (C, 7 H, aromatic), 5.3 (t, 1 H, α-proton of glutamate), 4.75 (5, 2 H, C^9-CH_2), 4.15 (s, 2 H, 2-butyne methylene), 2.0–2.5 (c, 4 H, glutamic), 1.48 (s, 3 H, 2-butyne). Anal. ($C_{25}H_{25}N_5O_6$) C, H, N.

N^{10}-(Cyclopropylmethyl)-5,8-dideazafolic Acid (3). A suspension of 508 mg (2 mmol) of the 6-(bromomethyl)quinazoline **5**, 752 mg (2 mmol) of the amine **9**, and 80 mg of MgO in 10 mL of DMAc was stirred at 100–105 °C for 18 h. The reaction mixture, which became a clear solution during this period, was poured over 100 g of crushed ice, stirred, and filtered. The white solid 11 thus obtained was washed several times with water, dried in vacuum, and dissolved in 20 mL of a mixture of CH_2Cl_2 and MeOH. This solution was applied on a silica gel column, which was eluted with 10% MeOH in CH_2Cl_2. The fractions corresponding to the diester **11** were pooled and evaporated to dryness: yield 800 mg; mp 162–169 °C. Hydrolysis of 11 (700 mg) was carried out in a similar manner as described for the diester **10**: yield 625 mg; mp 239–235 °C dec; NMR (TFA) δ 7.6–8.4 (c, aromatic), 5.03 (s, 2 H, C^9-CH_2), 3.82 (c, 2 H, cyclopropylmethyl), 2.1–2.9 (c, 5 H, glutamate, cyclopropyl), 0.75, 0.40 (c, 4 H, cyclopropyl). Me_4Si was used as an internal standard in this determination. The UV spectral data of **3** were identical to those reported previously.[24]

General Methods for the Preparation of the Poly-γ-glutamyl Derivatives of PDDF. (a) Esterification of Merrifield Chloromethyl Resin. One equivalent of 2% cross-linked resin with a chloride equivalent of 1 mmol/g was heated with a solution of 2 equiv of the cesium salt of *N-tert*-Boc-L-glutamic acid α-benzyl ester in DMF at 80 °C for 20 h and filtered. The resin was washed successively with water, ethanol, 1 M HOAc, water, and ethanol. After drying over P_2O_5 in vacuum at 25 °C for 24 h, a portion of the resin was hydrolyzed with a 1:1 mixture of HOAc and 6 N HCl under reflux for 18 h and quantitated spectrophotometrically by the ninhydrin method. This resin had an esterification value of 0.6 mmol of amino acid/g of resin.

(b) Deprotection of Resin Ester. Approximately 3 g of the above resin ester was suspended in 30 mL of CH_2Cl_2 with shaking for 10 min in a specially designed solid-phase reaction vessel, then filtered. A 25% solution of TFA in CH_2Cl_2 (60 mL) was introduced to the vessel, and the resin was shaken for 30 min and filtered. The deprotected resin ester was washed 3 times with 25-mL portions of CH_2Cl_2. After the washing, 60 mL of a 10% solution of triethylamine was introduced to the vessel, and the resin was allowed to mix for 10 min and filtered. The neutralized resin was washed 3 times with CH_2Cl_2 and kept ready for coupling.

(c) Preparation of the Mixed Anhydride of *N-tert*-Boc-L-glutamic Acid-α-Benzyl Ester and Coupling. For 3 mmol of the resin ester, 7.5 mmol of the glutamate derivative was used. In general, a 2.5-fold excess of the reagent relative to the glutamate derivative should be used. The glutamate derivative was dissolved in 50 mL of CH_2Cl_2 and cooled to 0 °C. To this solution was added 1.06 mL (9.375 mmol) of 4-methylmorpholine and, after 10 min, 0.98 mL (7.5 mmol) of isobutyl chloroformate. The reaction mixture was kept at 0 °C for 15 min more and then introduced to the solid-phase reaction vessel and mixed with the resin for 3 h and filtered. The resin was washed 3 times with CH_2Cl_2.

(d) Acetylation. A solution of 6 mL of acetic anhydride and 6 mL of triethylamine in 38 mL of CH_2Cl_2 was introduced to the reaction vessel and mixed with the coupled resin ester for 2 h and filtered. The resin was washed 3 times with 30-mL portions of CH_2Cl_2.

(e) Introduction of the Third and Successive Glutamate Moieties. The deprotection and neutralization of the acetylated resin were carried out exactly as described in part b. The mixed anhydride of *N-tert*-Boc-L-glutamic acid α-benzyl ester was made according to part c, coupled with the neutralized resin, and acetylated according to part d. The cycles of deprotection, neutralization, coupling, and acetylation were continued until the desired number of glutamate residues was introduced on the resin. After reaching the desired chain length, the resin was deprotected and kept ready for coupling with the mixed anhydride derived from N^{10}-propargyl-5,8-dideazapteroic acid and isobutyl chloroformate.

Preparation of the Mixed Anhydride 12. A solution of 348 mg (1 mmol) of **7** was made in 35 mL of Me_2SO by heating and then cooling to 25 °C. This solution was mixed with 20 mL of dry THF and placed in an ice bath. After 10 min, 0.14 mL (1.25 mmol) of 4-methylmorpholine followed by 0.13 mL (1.0 mmol) of isobutyl chloroformate were added, mixed, and kept at 25 °C for 30 min. The mixed anhydride solution **12** was then introduced to the reaction vessel containing the deprotected resin ester.

Cleavage and Purification of the Poly-γ-glutamyl Metabolites. After mixing the mixed anhydride **12** with the deprotected resin ester of the desired chain length for 18 h, the resin-bound product was filtered and washed successively with Me_2SO (30 × 30 mL), ethanol, and *p*-dioxane. A 1:1 mixture of *p*-dioxane and 2 N NaOH was deaerated by bubbling N_2 through it for 10 min, and this solution (20 mL) was introduced to the reaction vessel containing the resin-bound product and mixed vigorously for 1 h. The contents of the reaction vessel were filtered into a flask containing 200 mL of distilled water, and the filtrate was immediately adjusted to pH 7.5 with 1 N HCl. The solution was diluted to 500 mL with distilled water and applied to a DEAE-cellulose column (28 × 3 cm), which was eluted with a linear NaCl gradient from 0 to 0.5 M at pH 7.0 in 0.005 M phosphate buffer. Each fraction of the column was monitored for optical density at 250 nm, and the major fractions corresponding to the products were pooled and diluted 10 times with distilled water. The diluted solution was reapplied onto a fresh DEAE-cellulose column of the same size; the column was washed thoroughly with distilled water, and the product was eluted with 15% NH_4OH. The ammonia eluate was evaporated to a small volume (15 mL), and the yield of the product was estimated spectrophotometrically. These samples were stored in the freezer. Samples for NMR spectra were obtained by acidification of the aqueous solutions of the ammonium salt with 1 N HCl to pH 3.5 and collection of the precipitated solid by centrifugation. The precipitates were washed with water, recentrifuged, and dried over P_2O_5 in vacuum. The percent yield of each of the polyglutamyl metabolites based on the starting pteroic acid **7** is shown in Table I.

Biological Evaluation. Antimicrobial activities of all analogues were determined according to previously published procedures.[25,26] Thymidylate synthase assays were performed according to the procedure of Wahba and Friedkin.[27] The enzyme inhibitory activities of analogues in intact L1210 cells were determined as described previously.[28] Permeabilization of the cells with dextran sulfate was carried out according to the procedure described by Kucera and Paulus.[29] Briefly, L1210 cells were permeabilized by incubation at 4 °C for 20 or 40 min in the presence of 400 μg/mL Na-dextran sulfate (MW = 500 000; Pharmacia) followed by washing and resuspension. Enzyme activity was determined at pH 7.4 by measuring the tritium released into water from 1 μM [5-^{3}H]dUMP by 5×10^6 cells during 30 min at 37 °C. The assay mixture contained 0.4 mM (*d*)-1-L-methylenetetrahydrofolate.

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Registry No. **2**, 103202-11-5; **3**, 97280-35-8; **4**, 101248-36-6; **5**, 58677-08-0; **6**, 101248-37-7; **7**, 101248-32-2; **8**, 103202-12-6; **9**, 97280-20-1; **10**, 103202-13-7; **11**, 97280-27-8; PDDP-G_2, 95398-69-9; PDDP-G_3, 95398-70-2; PDDP-G_4, 95398-71-3; PDDP-G_5, 95398-

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72-4; ethyl *p*-aminobenzoate, 94-09-7; propargylbromide, 106-96-7; 2-butyn-1-ol, 764-01-2; methanesulfonyl chloride, 124-63-0; 2-butyn-1-ol mesylate, 61493-85-4; diethyl (*p*-aminobenzoyl)-L-glutamate, 13726-52-8; (bromomethyl)cyclopropane, 7051-34-5; *N*-*tert*-butoxycarbonyl-L-glutamic acid α-benzyl ester cesium salt, 64196-63-0; *N*-*tert*-butoxycarbonyl-L-glutamic acid α-benzyl ester, 30924-93-7; dihydrofolate reductase, 9002-03-3; thymidylate synthase, 9031-61-2.

Mitomycin C Analogues with Increased Metal Complexing Ability

Bhashyam S. Iyengar, Salah M. Sami, Takeo Takahashi,† Elizabeth E. Sikorski, William A. Remers,* and William T. Bradner‡

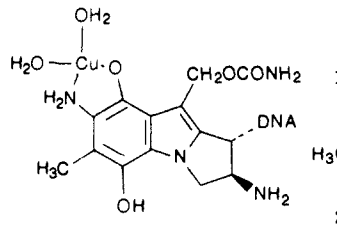
Department of Pharmaceutical Sciences, College of Pharmacy, University of Arizona, Tucson, Arizona 85721, and Department of Tumor Biology, Bristol-Myers Company, Syracuse, New York 13221-4755. *Received November 25, 1985*

Twenty-three new mitomycin C analogues designed to have increased metal complexing ability were synthesized and tested against P388 leukemia in mice. Their ability to complex Cu(II) was revealed by the shifts in their UV absorption spectra caused by this metal. One analogue was clearly more active than mitomycin C in the antitumor assay and two others had good activity. Correlation between antitumor activity and Cu(II) complexing ability was ambiguous. The most active compounds were either not complexers or they were complexers limited to the 2-(2-pyridyl)alkyl type substituent on N7. A variety of amino acid substituents on N7 showed only weak antitumor activity.

One of the ways in which mitomycins produce cytotoxic effects is based on their ability to generate reactive species, including hydrogen peroxide and hydroxyl radicals, when they are reduced and reoxidized by air.[1,2] These species are produced even when the mitomycins are bound to DNA, and they cause single-strand cleavage of the DNA. Formation of the reactive species is promoted by metal ions such as Cu(II) and inhibited by catalase, superoxide dismutase, general radical scavengers, and sequestering agent.[1] These observations suggest processes similar to those involved in strand cleavage by bleomycin or streptonigrin.[3,4] It has been proposed that the interaction of mitomycin hydroquinone with molecular oxygen in the presence of metal ions results in the formation of mitomycin semiquinone radical and superoxide radical, which undergo further transformation into hydrogen peroxide and hydroxyl radical.[1] The semiquinone radical has been detected by its electron paramagnetic resonance spectrum.[5] No structure has been proposed for the metal complex thought to be formed from mitomycin hydroquinone, although **1** is an obvious possibility with copper(II).

Our interest in mitomycin metal complexes was stimulated by a recent report that Cu(II) ions strongly promoted the strand scission of bacteriophage 0X174 by mitomycin C (**2**) and sodium dithionite.[6] Based on the possibility that mitomycin–metal complexes might be taken up by tumor cells and show an enhanced cytotoxic effect, we attempted the preparation of complexes from a variety of mitomycins and metals such as Zn(II), Cu(II), and Pt(II).[7] Mitomycin C did not form stable complexes, but rearranged to a 2-aminomitosene that was capable of complex formation. Analogues, such as the N^7-[2-(2-pyridyl)ethyl] derivative of mitomycin C (**4**), that contained basic nitrogens formed stable metal complexes without rearrangement. Testing of the resulting metal complexes against P388 leukemia in mice showed some antitumor activity, but it was much less than that of mitomycin C. On the other hand, the uncomplexed N^7-[2-(2-pyridyl)ethyl] derivative (**4**) had enhanced antitumor activity. Thus result suggested that decreased activity of the metal complexes might be caused by poor cell uptake, whereas the mitomycin analogue **4** with good potential complexing ability might be taken up adequately and then acquire metal ions from constituents within the cell.[7]

The present study is based on the idea that mitomycin C analogues with enhanced metal complexing ability might show improved antitumor activity. It is an extension of the observation that **4** and certain other mitomycin derivatives, for example the N^7-[(dimethylamino)ethyl] derivative, were very active in the P388 assay.[7,8] Because it was not obvious at the start which functional groups would be most effective in improving antitumor activity, we chose a variety of different types: some based closely on **4**, others with different heterocycles such as furan and imidazole, and still others with an array of aliphatic amines, ethers, and alcohols. One type of analogue receiving special attention was the amino acid substituent. Our previous preparation and testing of the glycine and



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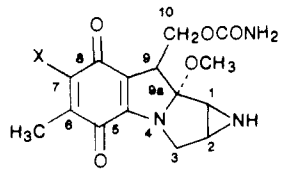
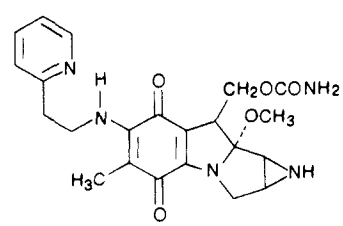


2, X = NH_2 (mitomycin C)
3, X = OCH_3 (mitomycin A)



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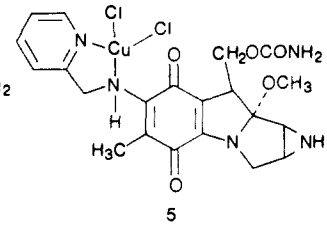

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†Visiting Professor from the Pharmaceutical Institute, Tohoku University, Sendai, Japan.
‡Bristol-Myers Company.

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