Syntheses and Thymidylate Synthase Inhibitory Activity of the Poly-γ-glutamyl Conjugates of *N*-[5-[*N*-(3,4-Dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl]-L-glutamic Acid (ICI D1694) and Other Quinazoline Antifolates†

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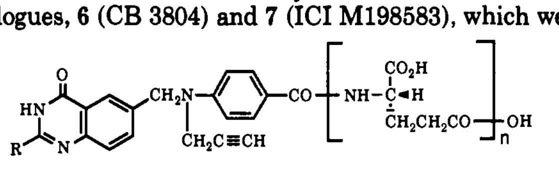
Thirteen poly-γ-glutamates derived from several novel antifolates have been synthesized by a convergent route. The syntheses of poly-γ-glutamyl conjugates of *N*-[5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl]-L-glutamic acid (**8**) (ICI D1694), 2-desamino-N^{10}-propargyl-5,8-dideazafolic acid (**6**), 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid (**7**), 2-desamino-2-methyl-N^{10}-propargyl-2′-fluoro-5,8-dideazafolic acid (**9**), and 2-desamino-2-methyl-4-chloro-N^{10}-propargyl-2′-fluoro-3,5,8-trideazafolic acid (**11**) are described. A key step in the route involves coupling of an α-*tert*-butyl-protected poly-γ-glutamate of the required chain length to the appropriate 5,8-dideazapteroic acid, obtained by carboxypeptidase G_2 cleavage of the parent monoglutamate, if available, or by chemical synthesis. Deprotection with trifluoroacetic acid in the final step gave the desired poly-γ-glutamyl antifolates as their trifluoroacetate salts. As inhibitors of thymidylate synthase, these polyglutamates were more potent in every case than the corresponding non-polyglutamylated drug.

Introduction

The quinazoline-based antifolate N^{10}-propargyl-5,8-dideazafolic acid **1** (CB 3717)[1] is a potent inhibitor of the enzyme thymidylate synthase (TS, EC 2.1.1.45).[2-5] Compound **1** has been shown to be a substrate for isolated mouse liver folylpolyglutamate synthetase (FPGS)[6] and to be metabolized intracellularly to poly-γ-glutamate derivatives **2–5** (predominantly the tetra and pentaglutamates).[7] These metabolites are very much more potent inhibitors of isolated mouse L1210 TS and human W1-L2 TS (100–200-fold) than the parent monoglutamate.[7-9] The polyglutamates of **1** are preferentially retained within L1210 cells.[7] Their ability to be retained is thought to be a function of the increased number of negatively charged carboxylates present on the extended peptide chain. Polyglutamylation may therefore play an important role in the cytotoxic activity of **1**. Although **1** showed promising antitumor activity in phase I and phase II trials,[10] its poor aqueous solubility resulted in unacceptable hepatic and renal toxicities,[11-13] and the drug was eventually withdrawn from the clinic. Further developmental work led to the discovery of the more water-soluble analogues, **6** (CB 3804) and **7** (ICI M198583), which were less active against TS (10- and 3-fold respectively) but significantly more cytotoxic to L1210 cells (10- and 40-



R \ n	1	2	3	4	5
NH_2	1	2	3	4	5
H	6	-	36	37	-
CH_3	7	31	32	33	34

† Part of this work has been presented in preliminary form: Bisset, G.; Pawelczak, K.; Jackman, A. L.; Dix Perkin, E. E.; Calvert, A. H.; Hughes, L. R. The Synthesis and Thymidylate Synthase (TS) Inhibitory Activity of the Poly-γ-glutamyl Derivatives of Some Analogues of N^{10}-Propargyl-5,8-dideazafolate (CB 3717). *Brit. J. Cancer* **1989**, *60*, 501.

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(1) Synonyms: PDDF; ICI 155,387; NSC 327182; *N*-[4-[*N*-[(2-amino-3,4-dihydro-4-oxo-6-quinazolinyl)methyl]prop-2-ynylamino]benzoyl]-L-glutamic acid.

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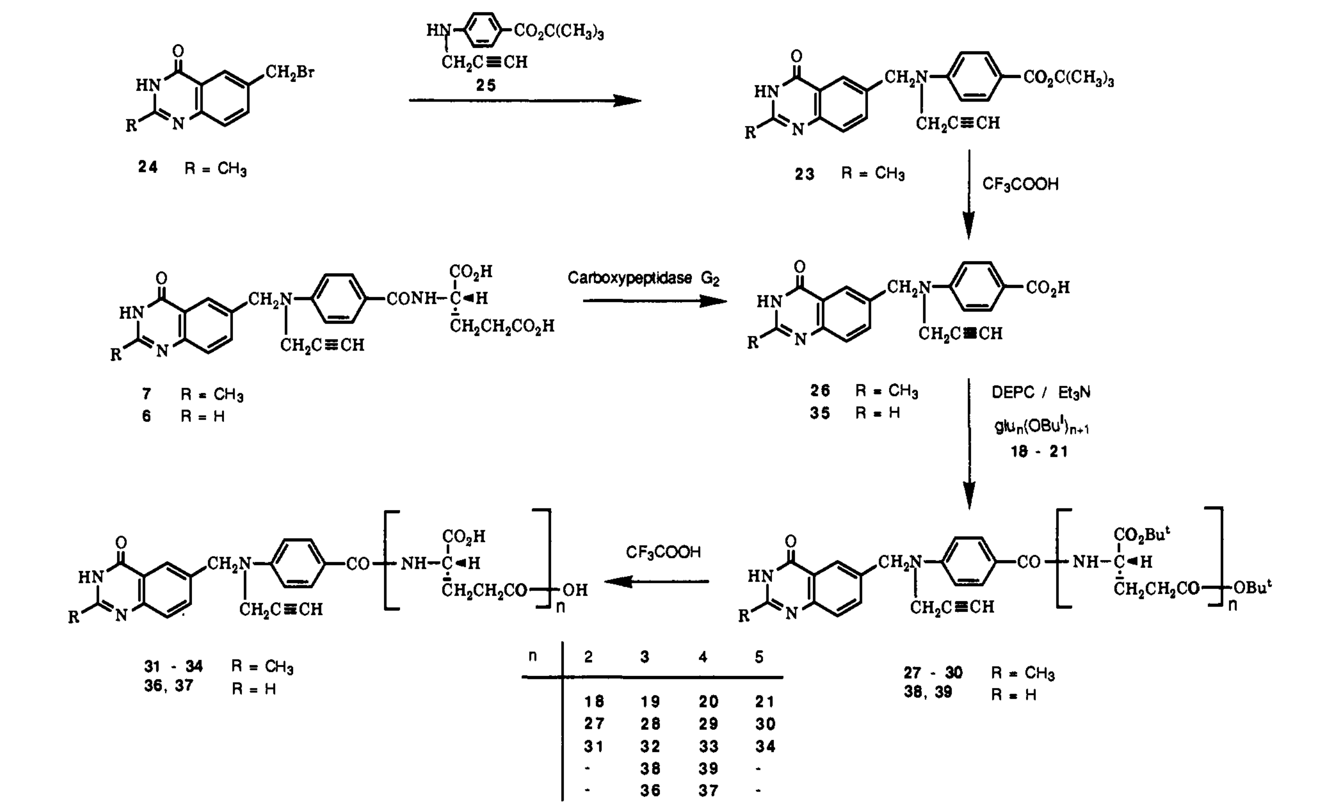
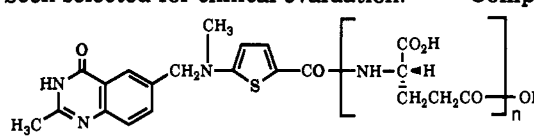
Scheme I



n	2	3	4	5
	18	19	20	21
	27	28	29	30
	31	32	33	34
	-	38	39	-
	-	36	37	-

fold).[5,14-18] Replacing the benzene ring of 7 with a thiophene ring and the 10-propargyl substituent with a methyl group produced compound 8 (ICI D1694),[19] which has been selected for clinical evaluation.[20-23] Compound



n	1	2	3	4	5	6
	8	47	48	49	50	51

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(19) Synonym: *N*-[5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl]-L-glutamic acid.

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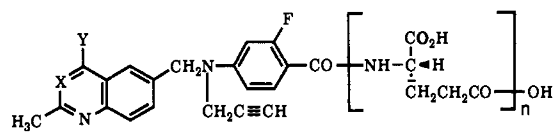
Table I. Preparation of "Desglutamyl" Intermediates by Carboxypeptidase G_2 Cleavage Method

compd	starting from	yield, %	mp, °C	mass spectra: m/z, M^+	HPLC purity, %	formula	analyses
26	**7**	99	228–230 dec	347	98	$C_{20}H_{17}N_3O_3{\cdot}0.5H_2O$	C,H,N
35	**6**	98	244–245 dec	333	95	$C_{19}H_{15}N_3O_3{\cdot}0.25H_2O$	C,H,N
40	**8**	94	185–186	330[a]	85	$C_{16}H_{15}N_3O_3S{\cdot}0.25H_2O$	C,H,N,S
52	**9**	95	235 dec	366[a]	96	$C_{20}H_{16}FN_3O_3{\cdot}1.1H_2O$	C,H,N,F
53	11	91	185–187 dec	383	95	$C_{21}H_{16}ClFN_2O_2{\cdot}0.5H_2O$	C,H,N

[a] $M^+ + 1$.

8 is both more water-soluble and considerably more cytotoxic in L1210 cell culture (IC_{50} = 0.007 μM, 600-fold) than **1**, despite being less potent against L1210 TS (K_i = 0.062 μM, 20-fold).[20,21,24] All these analogues were substrates for mouse liver FPGS.[5,17,25] Since polyglutamylation of **6–8** may be significant in antitumor activity, the inhibitory activity of their polyglutamates against the L1210 enzyme was investigated in vitro. In this paper we describe the syntheses and biological activity of some of the poly-γ-glutamyl derivatives of **6–8**. As further examples of the generality of the synthesis, we have included the preparation of the 2′-fluoro diglutamate **10** and the quinoline triglutamate **12**.



9	X = N	Y = OH	n = 1
10	X = N	Y = OH	n = 2
11	X = CH	Y = Cl	n = 1
12	X = CH	Y = Cl	n = 3

Chemistry

Several synthetic strategies have been developed for the polyglutamylation of folates and antifolates, employing either conventional solution-[8,26–28] or solid-phase peptide chemistry.[26,29] Recent advances in the area have included the use of α-*tert*-butyl *N*-[[(9-fluorenylmethoxy)oxy]carbonyl]-L-glutamate in conjunction with KH polyamide resin for chain elongation,[30] while Dunlap and co-workers have described a method based on the chemistry of folate azalactones.[31] Our own efforts have centered on devel-

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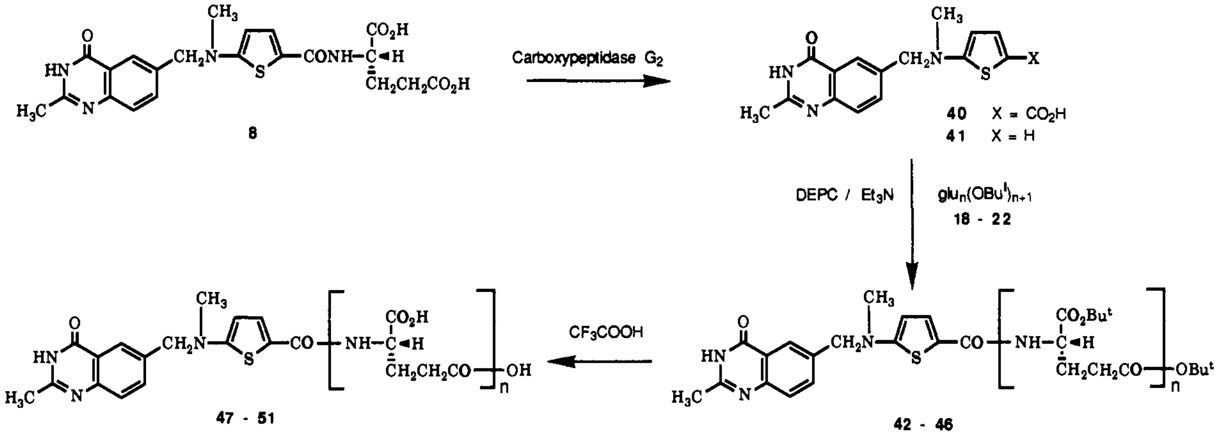
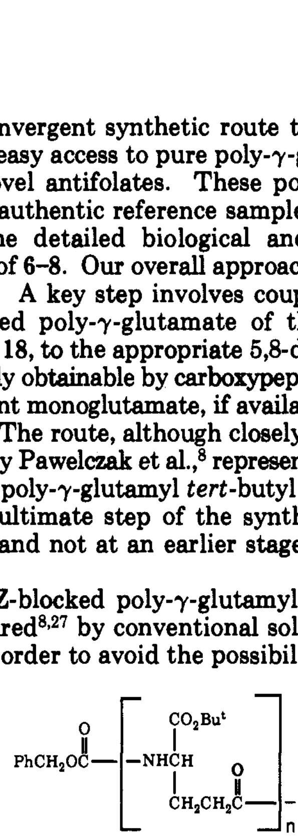
Scheme II



n	2	3	4	5	6
	18	19	20	21	22
	42	43	44	45	46
	47	48	49	50	51

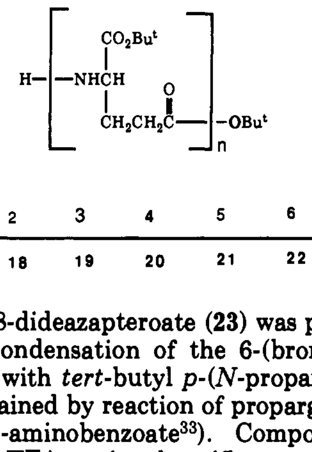
oping a convergent synthetic route that would provide quick and easy access to pure poly-γ-glutamates of a variety of novel antifolates. These polyglutamates were needed as authentic reference samples in studies to determine the detailed biological and pharmacological properties of **6**–**8**. Our overall approach is summarized in Scheme I. A key step involves coupling an α-*tert*-butyl-protected poly-γ-glutamate of the required chain length, e.g. **18**, to the appropriate 5,8-dideazapteroic acid, e.g. **26**, easily obtainable by carboxypeptidase G_2 cleavage[32] of the parent monoglutamate, if available, or by chemical synthesis. The route, although closely related to the one described by Pawelczak et al.,[8] represents an improvement in that the poly-γ-glutamyl *tert*-butyl ester is committed in the penultimate step of the synthesis of each polyglutamate and not at an earlier stage as in the existing procedure.

The CBZ-blocked poly-γ-glutamyl derivatives **13**–**17** were prepared[8,27] by conventional solution peptide synthesis. In order to avoid the possibility of γ → α trans-



n	2	3	4	5	6
	13	14	15	16	17

peptidation associated with alkaline carboxyl deprotection, the *tert*-butyl group was employed to protect the glutamyl α-carboxyls.[8,30] Hydrogenation of **13**–**17** over 10% palladium on charcoal afforded the poly-γ-glutamyl amines **18**–**22** in high yield. *tert*-Butyl 2-desamino-2-methyl-



n	2	3	4	5	6
	18	19	20	21	22

N^{10}-propargyl-5,8-dideazapteroate (**23**) was prepared by base-catalyzed condensation of the 6-(bromomethyl)-quinazoline **24**[18] with *tert*-butyl *p*-(*N*-propargylamino)-benzoate **25** (obtained by reaction of propargyl bromide with *tert*-butyl *p*-aminobenzoate[33]). Compound **23** was deprotected with TFA to give the trifluoroacetate salt of 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazapteroic acid (**26**), which could also be obtained by carboxypeptidase G_2 cleavage of **7** (Scheme I). The poly-γ-glutamyl *tert*-butyl esters (**27**–**30**) of **7** with a chain length of up to five glutamates were synthesized in good yield by condensing **26** with the required amine (**18**–**21**) using diethyl cyanophosphoridate as coupling reagent.[34] Each ester was purified by column chromatography. Removal of the *tert*-butyl protecting groups in the last step was accomplished with TFA to give polyglutamates **31**–**34** as their trifluoroacetate salts.

2-Desamino-N^{10}-propargyl-5,8-dideazapteroic acid (**35**) was obtained in 98% yield by carboxypeptidase G_2 cleavage of **6**[16] (Scheme I). The tri- and tetraglutamates, **36** and **37**, respectively, were synthesized by condensing **35** with the required amine, **19** or **20**, followed by TFA deprotection.

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Table II. Preparation of Polyglutamate *tert*-Butyl Esters

compd	*n*	starting from	yield, %	mp, °C	mass spectra: *m/z*, M+	HPLC purity, %	formula	analyses
27	2	**26**	63	107–108	774[a]	99	$C_{42}H_{55}N_5O_9\cdot0.5H_2O$	C,H,N
28	3	**26**	64	109–110	981[b]	98	$C_{51}H_{70}N_6O_{12}\cdot0.5H_2O$	C,H,N
29	4	**26**	55	112–113	1144	97	$C_{60}H_{85}N_7O_{15}\cdot0.5H_2O$	C,H,N
30	5	**26**	52	118–120	1329[a]	98	$C_{69}H_{100}N_8O_{18}$	C,H,N
38	3	**35**	58	126–127	945[a]	99	$C_{50}H_{68}N_6O_{12}\cdot0.5H_2O$	C,H,N
39	4	**35**	54	128–130	1130[a]	98	$C_{59}H_{83}N_7O_{15}\cdot0.5H_2O$	C,H,N
42	2	**40**	59	89–90	756[a]	100	$C_{38}H_{53}N_5O_9S\cdot0.6H_2O$	C,H,N,S
43	3	**40**	59	123–124	940	98	$C_{47}H_{68}N_6O_{12}S\cdot0.5H_2O$	C,H,N,S
44	4	**40**	55	135–136	1148[b]	100	$C_{56}H_{83}N_7O_{15}S\cdot0.5H_2O$	C,H,N,S
45	5	**40**	49	149–150	1312[a]	100	$C_{65}H_{98}N_8O_{18}S\cdot0.25H_2O$	C,H,N,S
46	6	**40**	44	110–111	1519[b]	100	$C_{74}H_{113}N_9O_{21}S\cdot H_2O$	C,H,N,S
54	2	**52**	62	115–117	792[a]	98	$C_{42}H_{54}FN_5O_9\cdot0.4H_2O$	C,H,N
55	3	**53**	60	105–108	994[a]	95	$C_{52}H_{69}ClFN_5O_{11}\cdot0.6H_2O$	C,H,N

[a] M+ + 1. [b] M+ + Na.

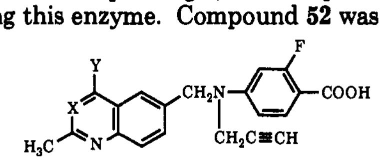
Table III. Preparation of Deprotected Polyglutamates

compd	*n*	starting from	yield, %	mp, °C	mass spectra: *m/z*, M+ + 1	HPLC purity, %	formula	analyses
10	2	**54**	92	110–112	624	98	$C_{30}H_{30}FN_5O_9\cdot CF_3COOH$	C,H,N
12	3	**55**	95	108–109	770	95	$C_{36}H_{37}ClFN_5O_{11}\cdot1.4CF_3COOH\cdot H_2O\cdot Et_2O$	C,H,N
31	2	**27**	97	147–148	606	99	$C_{30}H_{31}N_5O_9\cdot0.6CF_3COOH\cdot1.75H_2O$	C,H,N,F
32	3	**28**	96	143–144	735	98	$C_{35}H_{38}N_6O_{12}\cdot0.9CF_3COOH\cdot2H_2O\cdot Et_2O$	C,H,N,F
33	4	**29**	93	153–155	864	96	$C_{40}H_{45}N_7O_{15}\cdot0.6CF_3COOH$	C,H,N
34	5	**30**	92	160–164	993	93	$C_{45}H_{52}N_8O_{18}\cdot1.1CF_3COOH$	C,H,N
36	3	**38**	88	145–147	721	95	$C_{34}H_{36}N_6O_{12}\cdot0.5CF_3COOH$	C,H,N
37	4	**39**	93	151–153	850	96	$C_{39}H_{43}N_7O_{15}\cdot0.5CF_3COOH$	C,H,N
47	2	**42**	87	148–150	588	100	$C_{26}H_{29}N_5O_9S\cdot0.7CF_3COOH\cdot H_2O\cdot Et_2O$	C,H,N,S
48	3	**43**	92	150–152	717	100	$C_{31}H_{38}N_6O_{12}S\cdot0.9CF_3COOH\cdot Et_2O$	C,H,N,S
49	4	**44**	92	157–158	846	100	$C_{36}H_{43}N_7O_{15}S\cdot1.2CF_3COOH\cdot3Et_2O$	C,H,N,S
50	5	**45**	94	155–156	975	100	$C_{41}H_{50}N_8O_{18}S\cdot CF_3COOH\cdot3H_2O\cdot3Et_2O$	C,H,N,S
51	6	**46**	92	144–146	1126[a]	100	$C_{46}H_{57}N_9O_{21}S\cdot CF_3COOH\cdot3H_2O\cdot3Et_2O$	C,H,N,S

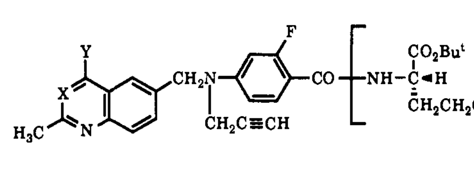
[a] M+ + Na.

Successful cleavage of the thiophene glutamate **8**[20] using carboxypeptidase G_2 requires careful control of the reaction conditions, particularly pH, temperature, and time. An exploratory overnight reaction run at 37 °C failed to give the desired pteroate analogue **40**, but instead compound **41** was isolated by filtration (Scheme II). However, investigation of the pH of the filtrate revealed the reaction had been run under acidic rather than neutral conditions. An improved cleavage procedure for bulk preparations involves dissolution of the substrate in Tris buffer at pH 10.4 and adjustment of the solution to pH 7.3 prior to addition of the enzyme. The cleavage was performed at 30 °C and HPLC monitoring of the reaction pathway showed maximal formation of the required carboxylate **40** between 1.5 to 2 h after addition of the carboxypeptidase G_2, although small amounts (5–15%) of decomposition product **41** were still consistently observed. The poly-γ-glutamyl *tert*-butyl esters **42–46** with a chain length of up to six glutamates were synthesized in good yield by condensing thenoic acid **40** with the required amine (**18–22**), again using diethyl cyanophosphoridate as the activating agent, followed by column chromatography to remove unwanted **41**. Removal of the quinazoline polyglutamate ester protecting groups in the last step was achieved with trifluoroacetic acid to give polyglutamic acids **47–51** as their trifluoroacetate salts. Compounds **47–51** are potential intracellular metabolites of **8** and have been employed as HPLC standards in the measurement of polyglutamate formation of **8** using ^3H labeled compound.[35]

The carboxypeptidase G_2 cleavage methodology proved to be convenient and versatile for the preparation of other novel antifolate polyglutamates. 2-Desamino-2-methyl-N^{10}-propargyl-2′-fluoro-5,8-dideazafolic acid (**9**)[36] was degraded to the corresponding 5,8-dideazapteroate **52** in 95% yield using this enzyme. Compound **52** was coupled with



52 X = N Y = OH
53 X = CH Y = Cl



54 X = N Y = OH n = 2
55 X = CH Y = Cl n = 3

amine **18** and subsequently deprotected with TFA to give the 2′-fluoro diglutamate **10**. Even 2-methyl-4-chloro-N^{10}-propargyl-2′-fluoro-3,5,8-trideazafolic acid (**11**)[37] was a good substrate for carboxypeptidase G_2, giving the corresponding 3,5,8-trideazapteroate **53** in 91% yield. This

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Table IV. Inhibitory Effects of Synthetic Polyglutamates on TS[a]

compd	total glu residues	TS $K_{iapp} \pm$ SE, nM	fold increase in TS inhibition
6	1	63.68 ± 2.89	
36	3	0.97 ± 0.06	66
37	4	0.63 ± 0.07	101
7	1	31.38 ± 1.92	
31	2	1.11 ± 1.92	28
32	3	0.40 ± 0.03	78
33	4	0.27 ± 0.03	115
34	5	0.40 ± 0.03	78
8	1	418	
47	2	24 ± 5	17
48	3	7.52 ± 0.49	56
49	4	4.69 ± 0.30	89
50	5	3.79 ± 0.17	110
51	6	3.7 ± 0.15	112
9	1	10.4 ± 1.6	
10	2	0.59 ± 0.03	18
11	1	63.7 ± 7	
12	3	1 ± 0.07	64

[a] L1210 enzyme.

was coupled with amine **19** and the *tert*-butyl groups removed as before with TFA to give the quinoline triglutamate **12**. The structure and purity of all compounds were established by elemental microanalysis (Tables I–III) and by NMR spectroscopy. Independent evidence for the structure of all compounds was also obtained by FAB mass spectrometry. All compounds were shown to be homogeneous by analytical HPLC.

Biological Evaluation

The antifolates listed in Table IV were tested as inhibitors of TS partially purified from L1210 mouse leukemia cells that overproduce TS due to amplification of the TS gene.[38] The partial purification and assay method used in this study was as previously described and used a (±)-5,10-methylenetetrahydrofolic acid concentration of 200 μM.[7,38] The K_i apparent was determined by using the Goldstein equation[39] applicable to tight-binding inhibitors. The data was fitted to the equation by a nonlinear least-squares regression analysis.[40]

Results and Discussion

The synthetic polyglutamates described above were tested as inhibitors of thymidylate synthase as part of a study to investigate the role of polyglutamylation in the activity of quinazoline antifolates targeted at TS. The results are given in Table IV.

The addition of one extra glutamate to **7** resulted in a 28-fold improvement in TS inhibitory activity. Further improvement, although less marked, was seen with the addition of two, three, or four extra glutamates (**32**, **33**, and **34**, respectively). These potential metabolites of **7** are therefore up to 2 orders of magnitude more potent as TS inhibitors than **7** itself. The addition of two or three extra glutamates to **6** gave a very similar improvement in TS inhibition. Indeed there is a remarkable similarity in the pattern of inhibition seen with these analogues when compared with published results for **1**.[7] This similarity also extends to the diglutamate derivative, **10**, of compound **9** (18-fold improvement) and to the structurally dissimilar quinoline triglutamate analogue **12** (64-fold improvement).

The biological activities of compounds **6** and **7** have been studied in further detail and are the subject of other communications.[5,16-18] Both compounds are substrates for isolated mouse liver FPGS[5,17,41] with activity similar to **1** (K_m values of 40–50 μM). The activity of **6** and **7** in in vitro and in vivo systems is entirely consistent with intracellular metabolism to polyglutamate forms that are not readily effluxed from the cell.[5,17] Studies utilizing tritiated **7** and its synthetic polyglutamates described above have indeed confirmed that polyglutamates are formed intracellularly within L1210 cells.[17]

The polyglutamates of **8** again show a pattern of improvement in TS inhibition very similar to that of analogues described above. Since the polyglutamation studies with tritiated **7** suggested that a very small amount of polyglutamates with chain length greater than pentaglutamate may be formed, we synthesized the hexaglutamate (**51**) of **8**. Clearly extending the polyglutamate chain beyond four or five extra glutamates does not improve TS inhibition any further.

The thiophene diglutamate **47** and tetraglutamate **49** have also been tested against isolated rat liver dihydrofolate reductase (DHFR). Neither of these derivatives was more potent an inhibitor of DHFR than **8** itself.[25] This is consistent with the very small improvement in DHFR inhibition seen with the polyglutamates of **1** or **6**.[5,7] As a substrate for FPGS **8** is very active, forming the diglutamate with a K_m of only 1.3 μM and a first-order rate constant (V_{max}/K_m) approximately 100-fold higher than that for compound **1**.[25]

Compound **8** is a very potent antitumor agent in vitro and in vivo[21,22,25] and this activity is thought to be due to rapid cellular uptake via the reduced-folate carrier and metabolism to polyglutamates that are not readily effluxed from the cell.[25] Rapid and extensive metabolism to polyglutamates (principally the tetraglutamate) has been confirmed by the HPLC analysis of extracts from cell cultures treated with tritiated **8**.[35] Thus the potency of the polyglutamates for TS described above and their cellular retentive properties probably accounts for the high cytotoxicity of compound **8** despite its relatively poor TS inhibitory activity.

In conclusion, we have developed a convergent synthetic route to poly-γ-glutamates of a variety of novel antifolates. The route is convenient and should have wide applicability in the synthesis of other folate and antifolate poly-γ-glutamate derivatives. As TS inhibitors, the polyglutamates are more potent in every case than the corresponding monoglutamate.

Experimental Section

N,N-Dimethylformamide (DMF) and *N,N*-dimethylacetamide (DMA) (Aldrich HPLC grades) were dried over 3-Å molecular sieves. Propargyl bromide was used as an 80% w/w solution in toluene (Aldrich). Tris(hydroxymethyl)aminomethane (Trizma base) was purchased from Sigma. TLC was performed on precoated sheets of silica $60F_{254}$ (Merck Art 5735). Spots were visualized with chlorine–tolidine reagent. Merck silica 60 (Art 7734) was used in gravity columns and Merck silica 60 (Art 15111) in low-pressure column chromatography. HPLC analyses were performed using a Waters Model 510 solvent delivery system,

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model 680 automated gradient controller, model U6K injector, and model 490 programmable wavelength detector set to monitor at 230 and 280 nm. Retention times were determined on a Trivector Trilab 3000 multichannel chromatography system. Separations were performed on a 15-cm × 0.46-cm column packed with 5-μM Spherisorb C6 (Phase Separations Ltd., U.K.) and eluted isocratically with different ratios of $MeOH/H_2O$ containing 1% HOAc. Electron-impact mass spectra were determined with a VG 7070H spectrometer and VG 2235 data system using the direct-insertion method, an ionizing voltage of 70 eV and a trap current of 100 μA, and an ion-source temperature of 160 °C. Fast atom bombardment mass spectra were determined with a VG ZAB-SE spectrometer, operating at 20 kV Cs^+ at 8 kV accelerating voltage in the source. NMR spectra were determined on Bruker WM250 and Bruker WH400 spectrometers. Field strengths are expressed in units of δ (ppm) relative to tetramethylsilane, and peak multiplicities are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; br s, broad singlet; br, broad signal; m, multiplet. Melting points were determined on a Kofler block and are uncorrected. Elemental analyses were determined by C.H.N. Analysis Limited, Leicester and Elemental Micro-Analysis Limited, Devon.

Preparation of CBZ-Blocked Di-, Tri-, Tetra-, Penta-, and Hexaglutamyl *tert*-Butyl Esters 13–17. The five CBZ-blocked poly-γ-glutamyl esters 13–17 were synthesized according to the method of Pawelczak et al.[8] Purifications were effected by chromatography on silica gel columns (Merck 15111) using either a 65% to 50% gradient of CH_2Cl_2 in EtOAc as the eluent (13–15) or 60% EtOAc in CH_2Cl_2 as eluent (16 and 17). The products (13–16) were crystallized from diethyl ether–petrol (1:4), while 17 was obtained as an oil.

Hydrogenolysis of CBZ-Blocked Polyglutamyl *tert*-Butyl Esters. Tetra-*tert*-butyl L-γ-Glutamyl-L-γ-glutamyl-L-glutamate (19). A solution of tetra-*tert*-butyl *N*-[*N*-[*N*-(benzyloxycarbonyl)-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamate (14) (16.8 g, 22 mmol) in EtOAc (300 mL) containing 10% Pd/C (2 g) in suspension was stirred under hydrogen at atmospheric pressure for 2.5 h, whereupon TLC showed the absence of starting material. The catalyst was filtered off and the filtrate concentrated in vacuo to give an oil (13.8 g, 99%), which crystallized on standing overnight. This solid was used without further purification.

The procedure was repeated with the appropriate CBZ-blocked *tert*-butyl polyglutamates 13 and 15–17 to yield the *tert*-butyl polyglutamates 18 and 20–22. These compounds had ^1H NMR spectra consistent with the assigned structures.

Carboxypeptidase G_2 Cleavage of Antifolate Diacids. 4-[*N*-[(3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]-*N*-prop-2-ynylamino]benzoic Acid (26). A stock solution of Tris buffer was prepared by dissolving tris(hydroxymethyl)aminomethane (12.11 g, 100 mmol) and $ZnCl_2$ (0.035 g, 0.26 mmol) in distilled H_2O (950 mL), adjusting the pH to 7.3 using 2 N HCl, and addition of more H_2O to a total volume of 1 L. *N*-[4-[*N*-[(3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]prop-2-ynylamino]benzoyl]-L-glutamic acid (7)[18] (0.25 g, 0.53 mmol) was dissolved in Tris buffer (40 mL) at 37 °C with shaking. The reaction was initiated by the addition of carboxypeptidase G_2 (80 μL of a stock solution, 1000 units/mL)[32] and the change in absorbance at 320 nm recorded on an SP8-150 double-beam spectrophotometer (Pye-Unicam). After 1.5 h the mixture was cooled in ice and adjusted to pH 4 with glacial HOAc. The precipitate was filtered off, washed with H_2O (2 × 50 mL), and dried in vacuo over P_2O_5 to give 26 as a white powder: 0.18 g (99%); mp 228–230 °C; NMR (Me_2SO-d_6) δ 2.33 (s, 3 H, C^2-CH_3), 3.26 (t, 1 H, C≡CH), 4.36 (d, 2 H, CH_2C≡C), 4.80 (s, 2 H, CH_2N), 6.82 (d, J = 8.0 Hz, 2 H, benzene 3′,5′-H), 7.55 (d, J = 8.2 Hz, 1 H, quinazoline 8-H), 7.69 (dd, J = 8.5 Hz, 1 H, quinazoline 7-H), 7.75 (d, J = 8.0 Hz, 2 H, benzene 2′,6′-H), 7.94 (d, 1 H, quinazoline 5-H), 12.21 (bd s, 1 H, lactam NH). Microanalytical and mass spectral data are given in Table I.

Compounds **35**, **52**, and **53** were prepared in a similar way from **6**,[16] **9**,[36] and **11**,[37] respectively, and had ^1H NMR spectra consistent with the assigned structures. Yields and analytical data are collected in Table I.

5-[*N*-[(3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]-*N*-methylamino]thenoic Acid (40). Compound 40 was obtained by the carboxypeptidase G_2 method described above except that the substrate starting material, 8,[20] was dissolved in Tris buffer at pH 10.4 and the solution adjusted to pH 7.3 using 2 N HCl. The cleavage was performed at 30 °C and the progress of the reaction was monitored using HPLC (solvent system; 60% MeOH/40% solution containing 1% HOAc in H_2O: the detector was set to measure absorbance at 226 nm). In a typical experiment, 8 (0.25 g, 0.55 mmol) was treated with carboxypeptidase G_2 (100 μL, 100 units of a stock solution) and the reaction shaken for 2 h and then acidified to pH 4 with 2 N HCl. The product was isolated by filtration, washed with water (4 × 25 mL), and dried in vacuo over P_2O_5, affording **40** as a light blue/green powder (0.17 g, 94%): NMR (Me_2SO-d_6) δ 2.33 (s, 3 H, C^2-CH_3), 3.07 (s, 3 H, N-CH_3), 4.69 (s, 2 H, CH_2N), 6.03 (d, J = 4.3 Hz, 1 H, thiophene 4′-H), 7.40 (d, J = 4.3 Hz, 1 H, thiophene 3′-H), 7.57 (d, J = 8.3 Hz, 1 H, quinazoline 8-H), 7.67 (dd, J = 8.3, 1.8 Hz, 1 H, quinazoline 7-H), 7.93 (d, J = 1.4 Hz, 1 H, quinazoline 5-H), 12.24 (s, 1 H, lactam NH). Microanalytical and mass spectral data are given in Table I.

2-[*N*-[(3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]-*N*-methylamino]thiophene (41). Compound 41 was isolated as the major product when 8 was cleaved using the methodology described for the preparation of **26**. The resulting precipitate was isolated by filtration, washed with water (4 × 25 mL), and dried in vacuo at 60 °C overnight, giving a light blue/green powder: mp 194–195 °C; NMR (Me_2SO-d_6) δ 2.33 (s, 3 H, C^2-CH_3), 2.91 (s, 3 H, N-CH_3), 4.52 (s, 2 H, CH_2N), 5.96 (dd, J = 3.6, 1.2 Hz, 1 H, thiophene 3′-H), 6.57 (dd, J = 5.4, 1.3 Hz, 1 H, thiophene 5′-H), 6.72 (dd, J = 5.3, 3.7 Hz, 1 H, thiophene 4′-H), 7.54 (d, J = 8.3 Hz, 1 H, quinazoline 8-H), 7.67 (dd, J = 8.3, 1.9 Hz, 1 H, quinazoline 7-H), 7.96 (s, 1 H, quinazoline 5-H), 12.22 (s, 1 H, lactam NH); MS m/z 285 (M^+). Anal. ($C_{15}H_{15}N_3OS \cdot 0.6H_2O$) C, H, N.

***tert*-Butyl 4-(Prop-2-ynylamino)benzoate (25).** *tert*-Butyl 4-aminobenzoate (19.3 g, 100 mmol),[33] propargyl bromide (80% solution in toluene, 16.4 g, 110 mmol), anhydrous K_2CO_3 (20 g, 145 mmol), and DMF (50 mL) were stirred under an argon atmosphere at 50 °C in the dark. After 3.5 h the solvent was removed in vacuo and the residue diluted with CH_2Cl_2 (350 mL) and then filtered. The filtrate was washed with H_2O (2 × 200 mL), dried (Na_2SO_4), and filtered once again. To the organic phase was added silica gel (60 g, Merck Art 7734) and the solvent removed in vacuo. The impregnated silica was applied to a column of silica gel (Merck Art 15111) and the column eluted with EtOAc (15%)/hexane (85%). Appropriate fractions were combined and concentrated in vacuo to give, after recrystallization from petrol, the desired product **25** as yellow needles: 10.6 g (46%); mp 91–92 °C; NMR (Me_2SO-d_6) δ 1.50 (s, 9 H, $C(CH_3)_3$), 3.12 (t, J = 2.4 Hz, 1 H, C≡CH), 3.93 (dd, J = 6.0, 2.4 Hz, 2 H, CH_2C≡C), 6.64 (d, J = 8.8 Hz, 2 H, aromatic 3′,5′-H), 6.78 (t, J = 6.0 Hz, 1 H, NH), 7.66 (d, J = 8.7 Hz, 2 H, aromatic 2′,6′-H); MS m/z 231 (M^+). Anal. ($C_{14}H_{17}NO_2$) C, H, N.

***tert*-Butyl 4-[*N*-[(3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]-*N*-prop-2-ynylamino]benzoate (23).** A solution of *tert*-butyl 4-(prop-2-ynylamino)benzoate (**25**) (7.70 g, 33 mmol), 6-(bromomethyl)quinazoline **24**[18] (7.97 g, 32 mmol), and dry $CaCO_3$ (4 g, 40 mmol) in DMA (50 mL) was stirred at 50 °C in the dark. After 17 h the $CaCO_3$ was filtered off and the filtrate concentrated in vacuo to give a brown oil. The oil was partitioned between EtOAc (500 mL) and dilute ammonium hydroxide solution (H_2O/18 N NH_3 10:1) (250 mL) and the EtOAc layer separated and washed with more dilute NH_4OH (2 × 250 mL) and then H_2O (250 mL). The EtOAc layer was separated, dried (Na_2SO_4), and reduced in volume to 200 mL in vacuo. After the mixture was cooled in ice the precipitate was collected by filtration, washed with cold EtOAc (2 × 25 mL), and dried in vacuo to give a white powder: 6.06 g (48%); mp 214 °C; NMR (Me_2SO-d_6) δ 1.49 (s, 9 H, $CO_2C(CH_3)_3$), 2.33 (s, 3 H, C^2-CH_3), 3.24 (t, J = 2.1 Hz, 1 H, C≡CH), 4.35 (d, J = 2.1 Hz, 2 H, CH_2C≡C), 4.79 (s, 2 H, CH_2N), 6.82 (d, J = 9.0 Hz, 2 H, benzene 3′,5′-H), 7.54 (d, J = 8.4 Hz, 1 H, quinazoline 8-H), 7.67 (dd, J = 8.1, 2.0 Hz, 1 H, quinazoline 7-H), 7.71 (d, J = 8.9 Hz, 2 H, benzene 2′,6′-H), 7.93 (d, J = 1.8 Hz, 1 H, quinazoline 5-H), 12.19 (s, 1 H, NH); MS m/z 403 (M^+). Anal. ($C_{24}H_{25}N_3O_3 \cdot {}^1/_4H_2O$) C, H, N.

4-[*N*-[(3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]-*N*-prop-2-ynylamino]benzoic Acid (26) Trifluoro-

acetate Salt. The foregoing *tert*-butyl ester **23** (1 g, 2.48 mmol) was dissolved in CF_3COOH (10 mL). After the reaction mixture was stirred under N_2 at ambient temperature and in the dark for 10 min, the solution was concentrated under reduced pressure. The yellow oily residue was triturated with EtOAc (50 mL), and the precipitate was filtered off and washed well with petrol to give a pale yellow powder: 1.11 g (97%); mp >300 °C; NMR (Me_2SO-d_6) δ 2.41 (s, 3 H, C^2-CH_3), 3.24 (t, J = 1.9 Hz, 1 H, C≡CH), 4.36 (d, J = 1.9 Hz, 2 H, CH_2C≡C), 4.82 (s, 2 H, CH_2N), 6.83 (d, J = 9.0 Hz, 2 H, benzene 3',5'-H), 7.59 (d, J = 8.4 Hz, 1 H, quinazoline 8-H), 7.75 (d, J = 9.0 Hz, 3 H, benzene 2',6'-H, quinazoline 7-H), 7.99 (d, J = 1.7 Hz, 1 H, quinazoline 5-H); MS m/z 348 (M^+ + 1). Anal. ($C_{19}H_{15}N_3O_3 \cdot CF_3CO_2H$) C, H, N.

Preparation of Antifolate Polyglutamate *tert*-Butyl Esters. Tetra-*tert*-butyl *N*-[*N*-[*N*-[5-[*N*-[(3,4-Dihydro-2-methyl-4-oxoquinazolin-6-yl)methyl]-*N*-methylamino]-2-thenoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamate (43). Thenoic acid **40** (0.329 g, 1 mmol) and tetra-*tert*-butyl L-γ-glutamyl-L-γ-glutamyl-L-glutamate (**19**) (0.944 g, 1.5 mmol) were dissolved in dry DMF (15 mL) at room temperature, and to this solution was added diethyl cyanophosphoridate (0.359 g, 2.2 mmol) and then Et_3N (0.222 g, 2.2 mmol). The mixture was stirred under nitrogen and in the dark for 2 h and then diluted with EtOAc (100 mL) and H_2O (100 mL). The water layer was separated and extracted with EtOAc (2 × 100 mL). The combined EtOAc extracts were washed with 10% aqueous citric acid (2 × 50 mL), saturated $NaHCO_3$ (100 mL), and dilute NaCl (100 mL), then dried (Na_2SO_4), filtered, and concentrated in vacuo. The residue was purified by chromatography on a silica gel column (Merck 15111) using EtOAc and then 1% MeOH in EtOAc as the eluent. The crude product **43** was crystallized from CH_2Cl_2/petrol, giving a white powder: 0.555 g (59%); mp 123–124 °C; NMR (Me_2SO-d_6) δ 1.38, 1.40 (2 × s, 36 H, $C(CH_3)_3$), 1.72, 1.87, 1.95 (3 × m, 6 H, CH_2^{β}), 2.16, 2.24 (2 × t, 6 H, CH_2^{γ}), 2.34 (s, 3 H, 2-CH_3), 3.04 (s, 3 H, N-CH_3), 4.07, 4.20 (2 × m, 3 H, CH^{α}), 4.66 (s, 2 H, CH_2N), 5.99 (d, J = 4.2 Hz, 1 H, thiophene 4'-H), 7.57 (m, 2 H, thiophene 3'-H + quinazoline 8-H), 7.66 (dd, J = 8.4, 2.0 Hz, 1 H, quinazoline 7-H), 7.94 (d, J = 1.7 Hz, 1 H, quinazoline 5-H), 8.14 (m, 3 H, CONH), 12.25 (s, 1 H, lactam NH); MS m/z 940 (M^+). Anal. ($C_{47}H_{68}N_6O_{12}S \cdot 0.5H_2O$) C, H, N, S.

The procedure was repeated with the appropriate primary amines **18–22** and appropriate thenoic or benzoic acids **26**, **35**, **40**, **52**, and **53** to give the coupled antifolate *tert*-butyl esters **27–30**, **38**, **39**, **42**, **44–46**, **54**, and **55**. Yields and mass spectral and analytical data of these products are given in Table II. The ^1H NMR spectra of these compounds were consistent with the assigned structures.

Preparation of Antifolate Polyglutamates. *N*-[*N*-[*N*-[5-[*N*-[(3,4-Dihydro-2-methyl-4-oxoquinazolin-6-yl)methyl]-*N*-methylamino]-2-thenoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamic Acid Trifluoroacetate Salt (48). A solution of **43** (0.150 g, 0.16 mmol) in TFA (10 mL) was stirred at room temperature for 1 h in the dark and under a nitrogen atmosphere. The solution was then concentrated in vacuo and the residue triturated with anhydrous Et_2O (30 mL). The solid was isolated by filtration, washed with Et_2O (4 × 10 mL), and dried in vacuo over P_2O_5, giving a pale yellow powder: 0.131 g, (92%); mp 150–152 °C; NMR (Me_2SO-d_6) δ 1.75–2.00 (3 × m, 6 H, CH_2^{β}), 2.18, 2.25 (2 × t, 2 H, 4 H, CH_2^{γ}), 2.38 (s, 3 H, 2-CH_3), 3.04 (s, 3 H, N-CH_3), 4.16, 4.28 (2 × m, 2 H, 1 H, CH^{α}), 4.67 (s, 2 H, CH_2N), 5.99 (d, J = 4.2 Hz, 1 H, thiophene 4'-H), 7.58 (2 × d, 2 H, thiophene 3'-H + quinazoline 8-H), 7.70 (dd, J = 8.4 Hz, 1 H, quinazoline 7-H), 7.96 (d, 1 H, quinazoline 5-H), 8.15 (m, 3 H, CONH), 12.46 (bd, COOH); MS m/z 717 (M^+ + 1). Anal. ($C_{31}H_{36}N_6O_{12}S \cdot 0.9CF_3COOH \cdot Et_2O$) C, H, N, S.

The procedure was repeated with the appropriate *tert*-butyl-protected polyglutamates to yield the antifolate poly-γ-glutamates **10**, **12**, **31–34**, **36**, **37**, **47** and **49–51**, all of which had ^1H NMR spectra consistent with the assigned structures. Yields and analytical data are gathered in Table III.

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Supplementary Material Available: ^1H NMR spectral data of "desglutamyl" compounds **26**, **35**, **52**, **53**, *tert*-butyl-protected polyglutamate esters **27–30**, **38**, **39**, **42–46**, **54**, **55**, and polyglutamate trifluoroacetate salts **10**, **12**, **31–34**, **36**, **37**, **47–51** (3 pages). Ordering information is given on any current masthead page.

Antitumor Agents. 123.[†] Synthesis and Human DNA Topoisomerase II Inhibitory Activity of 2'-Chloro Derivatives of Etoposide and 4β-(Arylamino)-4'-*O*-demethylpodophyllotoxins

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The 2'-chloro derivatives of etoposide and 4β-(arylamino)-4'-*O*-demethylpodophyllotoxins have been synthesized and evaluated for their inhibitory activity against the human DNA topoisomerase II as well as for their activity in causing cellular protein-linked DNA breakage. The results showed that none of the compounds are active as a result of the C-2' chloro substitution on ring E. This would suggest that the free rotation of ring E is essential for the aforementioned enzyme inhibitory activity. In addition, these 2'-chloro derivatives showed no significant cytotoxicity (KB).

Etoposide (VP-16, **1**) shows significant clinical activity against small-cell lung cancer, testicular cancer, lymphoma, and leukemia.[2] It has been proposed that **1** and related compounds exert their lethal effects by the inhibition of

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† For part 122, see ref 1.

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