1, J = 1.11 Hz, H2), 1.97 (d, 3, J = 1.11 Hz, H15); exact mass (FAB) found 409.2036.

Acknowledgment. We are grateful to Drs. A. M. Thomas, R. Schoenleber, their staffs, and P. Johnson for help during the initial stages of this project. We also thank Prof. S. Mizushima of Nagoya University for the donation of an overproducing strain of *E. coli*, thus enabling us to purify large quantities of isoleucyl-tRNA synthetase.

Registry No. **3**, 40980-52-7; **7a**, 117407-59-7; **7b**, 117407-79-1; **7c**, 117407-80-4; **8**, 117407-60-0; **9a**, 117407-61-1; **9b**, 117407-81-5; **9c**, 117407-82-6; **9** (R_1 = Et_3Si, X = OBu), 117407-90-6; **10a**, 75452-47-0; **10b**, 117407-85-9; **10c**, 117407-86-0; **11**, 117407-62-2; **12**, 117438-17-2; **13a**, 117407-63-3; **13b**, 117407-83-7; **14a**, 117407-64-4; **14b**, 117407-84-8; **15a**, 117407-65-5; **15b**, 117407-93-9; **16** (isomer 1), 117407-66-6; **16** (isomer 2), 117467-95-5; **17a** (isomer 1), 117407-67-7; **17a** (isomer 2), 117556-67-9; **17b** (isomer 1), 117407-94-0; **17b** (isomer 2), 117556-63-5; **18a**, 117407-68-8; **18b**, 117467-91-1; **19a**, 117407-69-9; **19a** (lactol), 117407-96-2; **19b**, 117407-95-1; **20**, 117407-70-2; **21**, 117407-71-3; **21** (tris t-$BuMe_2Si$ ether), 117407-97-3; **22**, 117407-72-4; **22** (tris t-$BuMe_2Si$ ether), 117407-98-4; **23**, 117407-73-5; **24**, 117407-74-6; **25**, 117407-75-7; **27**, 117438-18-3; **27** (R_1 = Ph), 117467-94-4; **28**, 117556-66-8; **28** (R_1 = Ph), 117408-03-4; **29a**, 117407-76-8; **29b**, 117408-02-3; **30a**, 117467-90-0; **30b**, 117467-92-2; (*R*)-**31**, 117407-77-9; (*S*)-**31**, 117467-93-3; **32**, 117407-99-5; i (isomer 1), 117407-99-5; i (isomer 2), 117556-68-0; ii, 117408-00-1; iii, 117408-01-2; iv, 117407-91-7; v, 117407-92-8; $HO(CH_2)_8CO_2CH_3$, 34957-73-8; $MeSO_2O(CH_2)_8CO_2CH_3$, 117407-87-1; $AcS(CH_2)_8CO_2CH_3$, 117407-88-2; $HS(CH_2)_8CO_2CH_3$, 117407-89-3; $HC(SCH_3)_3$, 5418-86-0; $BuSCH_2SiMe_3$, 18236-28-7; $PhSCH_2SiMe_3$, 17873-08-4; methyl oleate, 112-62-9; isoleucyl-tRNA synthetase, 9030-96-0.

Quinazoline Antifolates Inhibiting Thymidylate Synthase: Synthesis of Four Oligo(L-γ-glutamyl) Conjugates of N^{10}-Propargyl-5,8-dideazafolic Acid and Their Enzyme Inhibition

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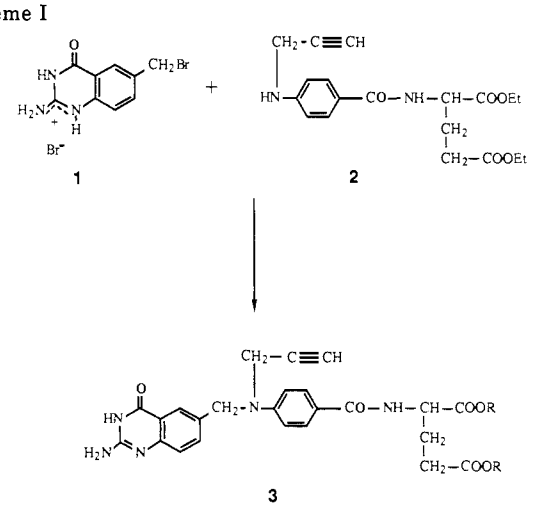
The synthesis is described of four oligo(γ-glutamyl) conjugates of N^{10}-propargyl-5,8-dideazafolic acid containing a total of two, three, four, and five L-glutamic acid residues. The *tert*-butyl group was chosen as the carboxyl protecting group in order to obviate the use of alkali and thus the possibility of γ → α transpeptidation. The starting material, di-*tert*-butyl glutamate, was coupled to *N*-(benzyloxycarbonyl)-L-glutamic acid α-*tert*-butyl ester via a mixed anhydride with isobutyl chloroformate. Hydrogenolysis of the benzyloxycarbonyl group in the product gave a carboxyl-protected diglutamate, which either was acylated with 4-[(benzyloxycarbonyl)amino]benzoyl chloride to give a protected aminobenzamide or was cycled further by using the above mixed anhydride/hydrogenolysis sequence into tri-, tetra-, and pentaglutamates. Each of the last named was also acylated, as above, to give a benzamide. The benzyloxycarbonyl group in the benzamides was removed by hydrogenolysis and the amino groups thus exposed were N-alkylated with propargyl bromide. The resulting proparglyamines were further alkylated with 2-amino-6-(bromomethyl)-4-hydroxyquinazoline hydrobromide to give the antifolate poly(*t*-Bu) esters. Deprotection with trifluoroacetic acid in the final step delivered the desired antifolates as their trifluoroacetate salts. The di- to pentaglutamates were, respectively, 31-, 97-, 171-, and 167-fold more inhibitory to WI-L2 human thymidylate synthase than the parent compound.

N^{10}-Propargyl-5,8-dideazafolic acid, CB3717,[1] is a novel tight-binding antifolate inhibitor[2] of the enzyme thymidylate synthase (EC 2.1.1.45) that has recently undergone clinical evaluation.[3] Polyglutamation is a known metabolic pathway for both natural folates[4] and classical antifolates,[5] resulting in their increased intracellular retention and enhanced binding to certain folate-metabolizing enzymes. The latter phenomenon has been amply demonstrated for thymidylate synthase.[6] Biochemical and pharmacological studies[7-9] of the polyglutamate derivatives of CB3717—extent of formation, transport characteristics, and role in the antitumor activity of the drug—led to a need for pure reference samples. The synthesis of four conjugates (**30–33**) of CB3717 and their inhibition of human TS is described herein. While our work was in progress, the preparation of these conjugates, by a different synthetic route, and a description of some of their biochemical properties was published by others.[10,11]

Scheme I



1 2 3

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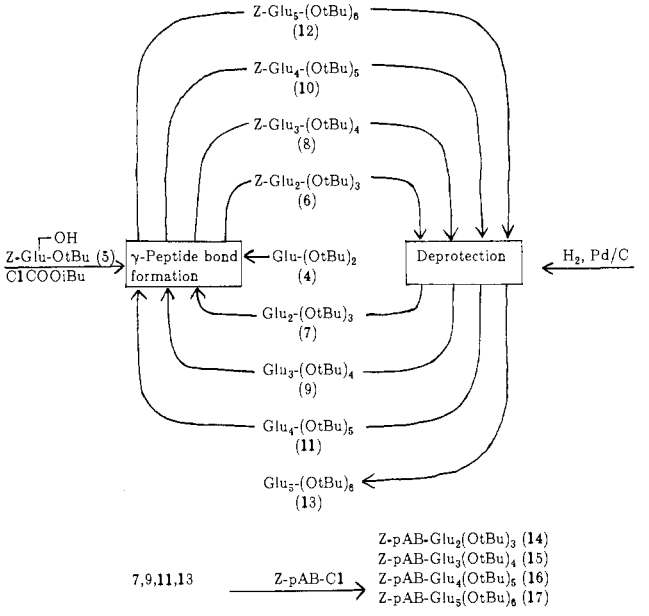
Chemistry

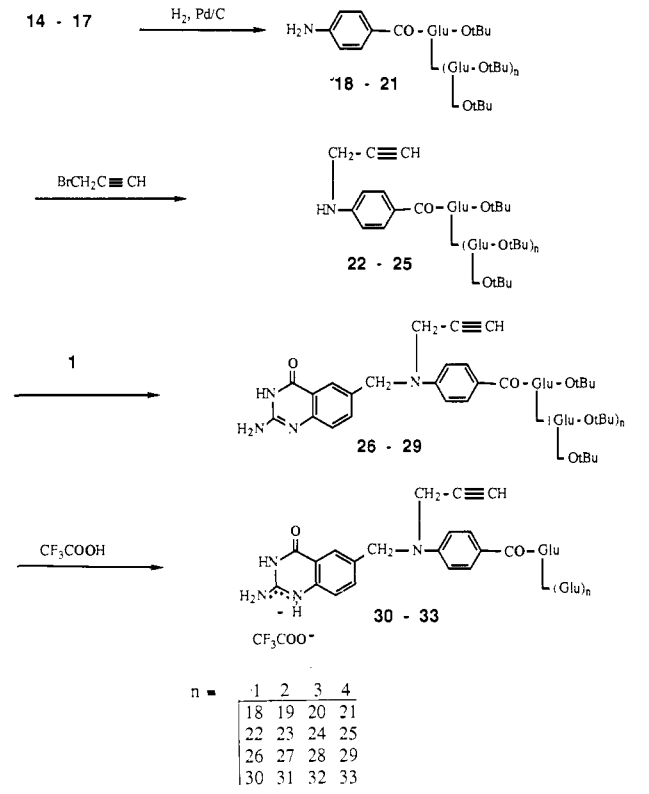
In planning our work we took note of the phenomenon of $\gamma \rightleftharpoons \alpha$ transpeptidation of glutamic acid derivatives, first observed in 1953[12] and subsequently confirmed.[13-16] This rearrangement is promoted by alkali, the use of which in the synthesis of compounds of this type received a cautionary note from Battersby and Robinson.[17] The *tert*-butyl carboxyl protecting group as a device to prevent transpeptidation was first advocated by Schwyzer in 1961.[18] Other workers went on to prove that transpeptidation does not occur when trifluoroacetic (TFA) is used to remove this group.[19] In a further study of TFA in this role, it was observed, parenthetically, that alkaline treatment of a trimethyl γ-diglutamate gave "eine Spur (höchstens 2%)" of α-glutamylglutamic acid.[20] Toward

Scheme II



Z = benzyloxycarbonyl, $C_6H_5CH_2OCO$; tBu = $(CH_3)_3C$;
pAB = para-aminobenzoyl, HN-C_6H_4-CO

Scheme III



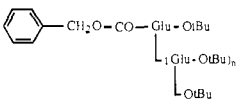
(1) Synonyms: ICI 155,387; NSC 327182; *N*-[4-[*N*-[(2-amino-4-hydroxy-6-quinazolinyl)methyl]prop-2-ynylamino]benzoyl]-L-glutamic acid.

(2) Jones, T. R.; Calvert, A. H.; Jackman, A. L.; Brown, S. J.; Jones, M.; Harrap, K. R. *Eur. J. Cancer* **1981**, *17*, 11.
(3) Calvert, A. H.; Alison, D. L.; Harland, S. J.; Robinson, B. A.; Jackman, A. L.; Jones, T. R.; Newell, D. R.; Siddik, Z, H.; Wiltshaw, E.; McElwain, T. J.; Smith, I. E.; Harrap, K. R. *J. Clin. Oncol.* **1986**, *4*, 1245.
(4) Reviewed in the following: (a) Kisliuk, R. L. In *Folate Antagonists as Therapeutic Agents*, Sirotnak, F. M., Burchall, J. J., Ensminger, W. D., Montgomery, J. A., Eds.; Academic Press: Orlando, 1984; Vol. 1, p 1. (b) McGuire, J. J.; Coward, J. K. In *Folates and Pterins*; Blakley, R. L., Benkovic, S. J., Eds.; John Wiley & Sons: New York, 1984; Vol. 1, p 135.
(5) (a) Rosenblatt, D. S.; Whitehead, V. M.; Vera, N.; Pottier, A.; Dupont, M.; Vuchich, M.-J. *Mol. Pharmacol.* **1978**, *14*, 1143. (b) McGuire, J. J.; Bertino, J. R. *Mol. Cellul. Biochem.* **1981**, *38*, 19. (c) Chabner, B. A.; Allegra, C. J.; Curt, G. A.; Clendeninn, N. J.; Baram, J.; Koizumi, S.; Drake, J. C.; Jolivet, J. *J. Clin. Invest.* **1985**, *76*, 907. (d) Moran, R. G.; Colman, P. D.; Rosowsky, R.; Forsch, R. A.; Chan, K. K. *Mol. Pharmacol.* **1985**, *27*, 156. (e) Goldman, I. D.; Matherly, L. H. *Pharmac. Ther.* **1985**, *28*, 77.
(6) (a) Kisliuk, R. L.; Gaumont, Y.; Baugh, C. M. *J. Biol. Chem.* **1974**, *249*, 4100. (b) Friedkin, M.; Plante, L. T.; Crawford, E. J.; Crumm, M. *J. Biol. Chem.* **1975**, *250*, 5614. (c) Jacobs, S. A.; Derr, C. J.; Johns, D. G. *Biochem. Pharmacol.* **1977**, *26*, 2310. (d) Dolnick, B. J.; Cheng, Y.-C. *J. Biol. Chem.* **1978**, *253*, 3563. (e) Szeto, D. W.; Cheng, Y.-C.; Rosowsky, A.; Yu, C. S.; Modest, E. J.; Piper, J. R.; Temple, C., Jr.; Elliott, R. D.; Rose, J. D.; Montgomery, J. A. *Biochem. Pharmacol.* **1979**, *28*, 2633. (f) Kisliuk, R. L.; Gaumont, Y.; Baugh, C. M.; Galivan, J. H.; Maley, G. F.; Maley, F. In *Chemistry and Biology of Pteridines*; Kisliuk, R. L., Brown, G. M., Eds.; Elsevier/North Holland: New York, 1979; p 431. (g) Maley, G. F.; Maley, F.; Baugh, C. M. *J. Biol. Chem.* **1979**, *254*, 7485. (h) Priest, D. G.; Mangum, M. *Arch. Biochem. Biophys.* **1981**, *210*, 118. (i) Kisliuk, R. L.; Gaumont, Y.; Lafer, E.; Baugh, C. M.; Montgomery, J. A. *Biochemistry* **1981**, *20*, 929. (j) Maley, G. F.; Maley, F.; Baugh, C. M. *Arch. Biochem. Biophys.* **1982**, *216*, 551. (k) Allegra, C. J.; Chabner, B. A.; Drake, J. C.; Lutz, R.; Rodbard, D.; Jolivet, J. *J. Biol. Chem.* **1985**, *260*, 9720. (l) Ueda, T.; Dutschman, G. E.; Nair, M. G.; DeGraw, J. I.; Sirotnak, F. M.; Cheng, Y.-C. *Mol. Pharmacol.* **1986**, *30*, 149.
(7) Sikora, E.; Newell, D. R.; Jackman, A. L.; Pawelczak, K.; Jones, T. R.; Calvert, A. H. *Brit. J. Cancer* **1986**, *54*, 178.
(8) Sikora, E.; Jackman, A. L.; Newell, D. R.; Harrap, K. R.; Calvert, A. H.; Jones, T. R.; Pawelczak, K.; Rzeszotarska, B. In *Chemistry and Biology of Pteridines* 1986; Cooper, B. A., Whitehead, V. M., Eds.; de Gruyter: Berlin, 1986; p 675.
(9) Manteuffel-Cymborowska, M.; Kamińska, B.; Grzelakowska-Sztabert, B. In *Chemistry and Biology of Pteridines 1986*; Cooper, B. A., Whitehead, V. M., Eds.; de Gruyter: Berlin, 1986; p 993.
(10) Cheng, Y.-C.; Dutschman, G. E.; Starnes, M. C.; Fisher, M. H.; Nanavathi, N. T.; Nair, M. G. *Cancer Res.* **1985**, *45*, 598.
(11) Nair, M. G.; Nanavati, N. T.; Nair, I. G.; Kisliuk, R. L.; Gaumont, Y.; Hsiao, M. C.; Kalman, T. I. *J. Med. Chem.* **1986**, *29*, 1754.
(12) Clayton, D. W.; Kenner, G. W. *Chem. Ind.* **1953**, 1205.
(13) Kovács, J.; Medzihradszky, K.; Bruckner, V. *Naturwiss.* **1954**, *41*, 450.
(14) Bruckner, V.; Kovács, J.; Medzihradszky, K. *Naturwiss.* **1955**, *42*, 96.
(15) Clayton, D. W.; Kenner, G. W.; Sheppard, R. C. *J. Chem. Soc.* **1956**, 371.
(16) Sondheimer, E.; Holley, R. W. *J. Am. Chem. Soc.* **1957**, *79*, 3767.
(17) Battersby, A. R.; Robinson, J. C. *J. Chem. Soc.* **1955**, 259.
(18) Schwyzer, R.; Dietrich, H. *Helv. Chim. Acta* **1961**, *44*, 2003.
(19) Kovacs, J.; Giannotti, R.; Kapoor, A. *J. Am. Chem. Soc.* **1966**, *88*, 2282.

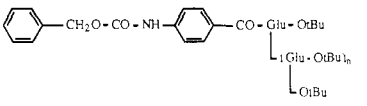
the synthesis (reviewed in ref 21; see also ref 22) of folate and antifolate polyglutamates, several workers have opted for the more commonly practiced solid-phase method and have used alkali in deprotection steps apparently unmindful of the danger it posed. Meienhofer and colleagues were the first to recognize the problem of $\gamma \rightarrow \alpha$ transpeptidation and chose the *tert*-butyl group in their synthesis of polyglutamates of folic acid.[23] This preference has been echoed in recent times by other researchers.[24-27] From this survey we were in no doubt that conventional solution peptide synthesis utilizing the *tert*-butyl group was more appropriate to our work.

The general synthetic approach is based on the synthesis of N^{10}-propargyl-5,8-dideazafolic acid (**3**, R = H) and its derivatives, which involved the propargylation of diethyl *N*-(4-aminobenzoyl)-L-glutamate to give the secondary amine, **2**, which was then alkylated with 2-amino-6-(bromomethyl)-4-hydroxyquinazoline hydrobromide (**1**) (Scheme I). Saponification of the diester product (**3**, R = Et) was the final step.[2,28-30] The extension of this approach to higher conjugates required the preparation of the appropriate (*tert*-butyl oligo(γ-glutamate) esters containing up to five units (**7**, **9**, **11**, and **13**, Scheme II). The starting material, di-*tert*-butyl glutamate (**4**), and each of the lowermost oligoglutamates (**7**, **9**, and **11**) were cycled (Scheme II) to the next higher by coupling *N*-(benzyloxycarbonyl)-L-glutamic acid α-*tert*-butyl ester (**5**)[31] followed by hydrogenolysis of the benzyloxycarbonyl group in the product. The intermediates **6**, **8**, **10**, and **12** were first described by Meienhofer et al.[23a] whose work we improve with shorter peptide coupling times, a simplified workup, and a better hydrogenation catalyst. Coward,[24] also following Meienhofer, had briefly characterized the amines **7**, **9**, **11**, and **13**. We did not, but immediately acylated them with 4-[(benzyloxycarbonyl)amino]benzoyl chloride (Z-pAB-Cl)[32] to give the benzamides **14–17**. Deprotection of **14–17** by catalytic hydrogenolysis (Scheme III) gave the primary amines **18–21** that were *N*-alkylated with propargyl bromide to give the secondary amines **22–25**. These, upon further alkylation with the bromide **1**, gave the antifolate polyesters **26–29**. Removal of the *tert*-butyl groups in the last step was accomplished with TFA to give the CB3717 oligoglutamic acids as their trifluoroacetate salts, **30–33**. The structure and purity of all compounds containing the propargyl group were established by elemental microanalysis (Tables II and III) and by NMR spectroscopy (Table IV). The final products **30–33** were additionally characterized by analytical HPLC and were found to be 97–98% pure (**30–32**) and 94% pure (**33**) (Table III). They were tested for their tight-binding inhibition of human WI-L2 thymidylate synthase.

Table I. Preparation of N-Protected Oligoglutamate Esters and Intermediates **14–17**



6,8,10,12

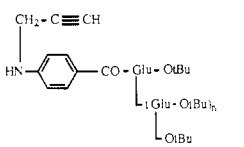


14 - 17

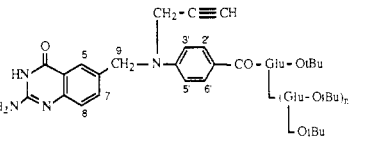
compd	*n* =	yield, %	mp, °C	formula[a]
6	1	94	86–88[b]	$C_{30}H_{46}N_2O_9$
8	2	82	78–79.5[c]	$C_{39}H_{61}N_3O_{12}$
10	3	91	78–80[d]	$C_{48}H_{76}N_4O_{15}$
12	4	80	100–102[e]	$C_{57}H_{91}N_5O_{18}$
14	1	90	129–130	$C_{37}H_{51}N_3O_{10}$
15	2	86	91–93[f]	$C_{46}H_{66}N_4O_{13}$
16	3	88	138–139	$C_{55}H_{81}N_5O_{16}$
17	4	72	100–102[f]	$C_{64}H_{96}N_6O_{19}$

[a] All compounds analyzed for C, H, and N within ±0.4%. [b] Lit.[21a] mp 83–84 °C. [c] Lit.[21a] mp 79.5–81 and 77.5–79 °C. [d] Lit.[21a] mp 84.5–85 and 87–89 °C. [e] Lit.[21a] mp 102–103.5 °C. [f] Not recrystallized, but chromatographically pure in four TLC systems (A, D, E, and F).

Table II. Preparation of Propargylamines **22–25** and Antifolate Polyesters **26–29**



22 - 25



26 - 29

compd	*n* =	scale, mmol	yield, %	mp, °C	formula[a]
22	1		40	153–153.5[b]	$C_{32}H_{47}N_3O_8$
23	2		64	65–66.5[c]	$C_{41}H_{62}N_4O_{11}$
24	3		39	129–130[d]	$C_{50}H_{77}N_5O_{14}$
25	4		37	95–98[c]	$C_{59}H_{92}N_6O_{17}$
26	1	4	71	145–148[c]	$C_{41}H_{54}N_6O_9 \cdot H_2O$[e]
27	2	2.5	72	150–153[c]	$C_{50}H_{69}N_7O_{12} \cdot H_2O$[e]
28	3	2.5	52	154–158[c]	$C_{59}H_{84}N_8O_{15} \cdot 2H_2O$[e]
29	4	1	43.5	135–137[f]	$C_{68}H_{99}N_9O_{18} \cdot 2H_2O$[e]

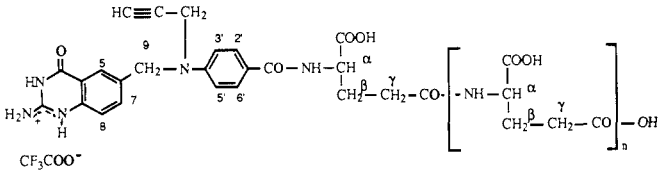
[a] All compounds analyzed for C, H, and N within ±0.4%, except %C for **29**: calcd, 59.76; found 59.31. [b] From AcOEt. [c] Amorphous solid, not recrystallized but chromatographically pure in four TLC systems (A, D, E, and F). [d] From AcOEt–petrol. [e] ^{1}H NMR spectrum showed the presence of water. [f] From $CHCl_3$–petrol.

Discussion

The inhibition of human thymidylate synthase by the polyglutamates **30–33** are listed in Table V as K_i apparent's with the value of the parent compound included as a positive control. All of the polyglutamates were more

(20) Kajtár, M.; Hollósi, M. *Acta Chim. Acad. Sci. Hung.* **1970**, *65*, 403.
(21) Krumdieck, C. L.; Tamura, T.; Eto, I. *Vitamins Hormones* **1983**, *40*, 45.
(22) D'Ari, L.; Rabinowitz, J. C. *Methods Enzymol.* **1985**, *113*, 169.
(23) (a) Meienhofer, J.; Jacobs, P. M.; Godwin, H. A.; Rosenberg, I. H. *J. Org. Chem.* **1970**, *35*, 4137. (b) Godwin, H. A.; Rosenberg, I. H.; Ferenz, C. R.; Jacobs, P. M.; Meienhofer, J. *J. Biol. Chem.* **1972**, *247*, 2266.
(24) Coward, J. K.; Parameswaran, K. N.; Cashmore, A. R.; Bertino, J. R. *Biochemistry* **1974**, *13*, 3899.
(25) (a) Drey, C. N. C.; Priestley, G. P. *J. Chem. Soc., Perkin Trans. 1* **1978**, 800. (b) Drey, C. N. C.; Priestley, G. P. *J. Chem. Res., Miniprint* **1979**, 3055.
(26) (a) Hynes, J. B.; Yang, Y. C. S.; McCue, G. H.; Benjamin, M. B. In *Folyl and Antifolyl Polyglutamates*; Goldman, I. D., Chabner, B. A., Bertino, J. R., Eds.; Plenum Press: New York, 1983; p 101. (b) Hynes, J. B.; Harmon, S. J.; Floyd, G. G.; Farrington, M.; Hart, L. D.; Gale, G. R.; Washtien, W. L.; Susten, S. S.; Freisheim, J. H. *J. Med. Chem.* **1985**, *28*, 209.
(27) Antonjuk, D. J.; Boadle, D. K.; Cheung, H. T. A.; Tran, T. Q. *J. Chem. Soc., Perkin Trans 1* **1984**, 1989.
(28) Jones, T. R.; Calvert, A. H.; Jackman, A. L.; Eakin, M. A.; Smithers, M. J.; Betteridge, R. F.; Newell, D. R.; Hayter, A. J.; Stocker, A.; Harland, S. J.; Davies, L. C.; Harrap, K. R. *J. Med. Chem.* **1985**, *28*, 1468.
(29) Jones, T. R.; Smithers, M. J.; Taylor, M. A.; Jackman, A. L.; Calvert, A. H.; Harland, S. J.; Harrap, K. R. *J. Med. Chem.* **1986**, *29*, 468.
(30) Jones, T. R.; Smithers, M. J.; Betteridge, R. F.; Taylor, M. A.; Jackman, A. L.; Calvert, A. H.; Davies, L. C.; Harrap, K. R. *J. Med. Chem.* **1986**, *29*, 1114.
(31) Pawelczak, K. Krzyzanowski, L.; Rzeszotarska, B. *Org. Prep. Proc. Int.* **1985**, *17*, 416.
(32) Krzyzanowski, L.; Rzeszotarska, B. *Org. Prep. Proc. Int.* **1985**, *17*, 83.

Table III. Preparation of Trifluoroacetate Salts of N^{10}-Propargyl-5,8-dideazafolic Acid Polyglutamates



30 - 33

compd	n =	scale, mmol	yield, %	mp, °C	formula[a]	HPLC purity,[b] %	HPLC capacity factor (k')
30	1	1	86	>300	$C_{29}H_{30}N_6O_9 \cdot 0.9CF_3COOH$	97.9 ± 0.1	12.3
31	2	1	90	>300	$C_{34}H_{37}N_7O_{12} \cdot 1.25CF_3COOH$	97.8 ± 1.2	7.1
32	3	0.3	89	>300	$C_{39}H_{44}N_8O_{15} \cdot CF_3COOH$	97.4 ± 0.7	4.9
33	4	0.2	87	>300	$C_{44}H_{51}N_9O_{18} \cdot 1.5CF_3COOH$	93.6 ± 0.6	3.5

[a] All compounds analyzed for C, H, and N within 0.4%, except %H for **33**: calcd, 4.54; found, 5.01. [b] Purity is the mean and standard deviation of five determinations.

inhibitory. A marked increase (31-fold) in inhibition was observed with the addition of the first glutamic acid to give the diglutamate **30**. Further but less dramatic increases were observed with the addition of two and three extra glutamates as in the compounds **31** and **32**, which were 97- and 171-fold more active than the parent. Addition of a fourth glutamate to give compound **33** had essentially no further effect. The most active compound was therefore the tetraglutamate **32**, which was over 2 orders of magnitude more inhibitory than the parent drug. These results are similar to those that we obtained from a study of murine enzyme[33] (both were obtained by using tight-binding inhibition kinetics[34]) and are in general agreement with those obtained by others in a study of another human enzyme[10] but wherein the pentaglutamate **33** was found to be the least active of the four. We have recently demonstrated that the tetra- and pentaglutamates **32** and **33** are formed in L1210 cells exposed to 10-propargyl-5,8-dideazafolic acid and have concluded that these metabolites contribute significantly to the in vitro cytotoxicity of the drug.[33] In conclusion, our results confirm previous experience[6] that polyglutamation affects binding to TS profoundly and this phenomenon therefore represents a most important aspect of the cellular pharmacology of a classical TS-inhibiting antifolate.

Experimental Section

Tetrahydrofuran (THF), dioxane, and diethyl ether were distilled from sodium and stored over sodium wire. *N,N*-Dimethylformamide (DMF) was azeotropically distilled and stored over activated (250 °C) 4-Å molecular sieves. *N,N*-Dimethylacetamide (DMA) was similarly dried. Petrol refers to petroleum ether, bp 60–80 °C. The hydrogenolysis catalyst was 10% Pd/C used at 20% of the peptide weight. Propargyl bromide was used as an 80% w/w solution in toluene (Aldrich). Reactions were monitored and the homogeneity of products were checked by TLC on silica 60 (Merck, Art 5553) with the following eluents: (A) $CHCl_3$–MeOH–AcOH (95:5:3), (B) AcOEt–CH_2Cl_2 (1:1), (C) AcOEt–CH_2Cl_2 (2:1), (D) $CHCl_3$–MeOH–concentrated NH_4OH_{aq} (6:20:1), (E) C_6H_6–pyridine–AcOH (20:2:1), (F) AcOEt–heptane (4:1), (G) $CHCl_3$–MeOH (10:1), (H) $CHCl_3$–CH_3OH (5:1). Spots were visualized with chlorine–tolidine reagent and, when amino compounds, additionally with ninhydrin. Merck silica 60 (Art. 9385) was used in gravity columns. Melting points were determined on a Boëtius apparatus and are uncorrected. ^1H NMR spectra were determined on Tesla BS567 (100 MHz) and Bruker WM 250 (250 MHz) spectrometers. Field strengths are expressed in units of δ (ppm), and peak multiplicities are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; br s, broad singlet; m, multiplet. Elemental analyses were performed by the Analytical Laboratory of the Institute of Organic Chemistry of the Polish Academy of Sciences, Warsaw. HPLC analyses were performed on a Waters Associates Chromatograph. Compounds were dissolved in 0.15 M $NaHCO_3$ at 1.0 mg·mL^{-1} and 10 μL analyzed. Separations were performed on a 10 cm × 0.46 cm Polygosil-60 5-μm C18 column eluted isocratically at 1.5 mL·min^{-1} with 0.1 M NaOAc pH 5/CH_3CN (9:1) and compounds detected by UV absorbance at 254 nm.

Tri-*tert*-butyl *N*-[*N*-(Benzyloxycarbonyl)-L-γ-glutamyl]-L-glutamate (6), Tetra-*tert*-butyl *N*-[*N*-[*N*-(Benzyloxycarbonyl)-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamate (8), Penta-*tert*-butyl *N*-[*N*-[*N*-[*N*-(Benzyloxycarbonyl)-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamate (10), and Hexa-*tert*-butyl *N*-[*N*-[*N*-[*N*-[*N*-(Benzyloxycarbonyl)-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamate (12). To a stirred solution of **5**[31] (1 mmol) and *N*-methylmorpholine (1 mmol) in THF (1 mL) cooled to −20 °C was added isobutyl chloroformate (1 mmol). After 10 min either a suspension of di-*tert*-butyl glutamate hydrochloride (**4**)[35] (1 mmol) in THF (1 mL) containing *N*-methylmorpholine (1 mmol) or a solution in THF of **7**, **9**, or **11** (1 mmol) (from hydrogenolysis of **6**, **8**, or **10**) was added. Stirring was continued for 10 min at −20 °C, and the mixture then allowed to warm to room temperature. *N*-Methylmorpholine hydrochloride was filtered off and the filtrate evaporated to dryness. The resulting crude oil was crystallized from diethyl ether–petrol. Yields and melting points of products chromatographically homogeneous in system A are given in Table I.

Hydrogenolysis of 6, 8, 10, and 12 To Produce 7, 9, 11, and 13. A solution of the protected peptide **6**, **8**, **10**, or **12** (1 mmol) in MeOH (3.5 mL) containing Pd/C in suspension was stirred under hydrogen for 30 min to 1 h, whereupon TLC (system A) showed the absence of starting material. The catalyst was removed by filtration and the filtrate evaporated to dryness. The resulting oil was used immediately in the next reaction.

Tri-*tert*-butyl *N*-[*N*-[4-[(Benzyloxycarbonyl)amino]-benzoyl]-L-γ-glutamyl]-L-glutamate (14), Tetra-*tert*-butyl *N*-[*N*-[*N*-[4-[(Benzyloxycarbonyl)amino]benzoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamate (15), Penta-*tert*-butyl *N*-[*N*-[*N*-[*N*-[4-[(Benzyloxycarbonyl)amino]benzoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamate (16), and Hexa-*tert*-butyl *N*-[*N*-[*N*-[*N*-[*N*-[4-[(Benzyloxycarbonyl)amino]benzoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamate (17). To a stirred solution of **7**, **9**, **11**, or **13** (1 mmol) and *N*-methylmorpholine (1 mmol) in dioxane (1 mL) kept at 20 °C was added 4-[(benzyloxycarbonyl)amino]benzoyl chloride[32] (1 mmol). Stirring was con-

(33) Sikora, E.; Jackman, A. L.; Newell, D. R.; Calvert, A. H. *Biochem. Pharmacol.* **1988**, *37*, 4047.
(34) Goldstein, A. *J. Gen. Physiol.* **1944**, *27*, 529.
(35) Kamber, B. *Helv. Chim. Acta* **1971**, *54*, 398.

Table IV. ^{1}H NMR Spectral Data of Propargylamines, Antifolate Polyesters, and Trifluoroacetate Salts of CB3717 Polyglutamates[a]

compd	spectrum freq, MHz	t-Bu (s)	Glu[b] $(CH_2^{\beta}$–$CH_2^{\gamma})_n$ (m)	propargyl H (t)	propargyl CH_2 (d)	Glu[c] $(CH^{\alpha})_n$ (m)	Glu[c] (CH^{γ}) (m)	CH_2[9] (s)	NH_2 (br s)	NH^{10} (t)	3',5' (d)	2',6' (d)	H^8 (d)	H^7 (dd)	H^5 (d)	amidic protons $(CONH)_n$ (m)	amidic protons ArCONH (d)
22	100	1.33	1.62–2.36	3.01	3.87	4.18				6.49	6.59	7.66				8.08	8.21
23	100	1.35	1.62–2.36	2.99	3.89	4.13				6.43	6.60	7.68				8.07	8.25
24	250	1.39	1.62–2.36	3.08	3.93	4.09–4.27				6.55	6.65	7.72				8.12	8.26
25	250	1.39	1.62–2.36	3.09	3.93	4.09–4.27				6.56	6.65	7.72				8.13	8.28
26	250	1.38	1.6–2.3	3.20	4.05		4.30	4.67	6.37		6.83	7.75	7.17	7.49	7.79	8.13	8.32
27	250	1.38	1.6–2.3	3.20	4.05		4.30	4.67	6.36		6.84	7.75	7.17	7.49	7.79	8.14	8.35
28	250	1.39	1.6–2.4	3.20	4.02		4.38	4.68	6.48		6.84	7.76	7.19	7.51	7.80	8.14	8.35
29	250	1.39	1.6–2.4	3.19	4.02		4.38	4.67	6.41		6.84	7.76	7.17	7.48	7.78	8.14	8.35
30	100		1.6–2.3	3.13	4.05		4.40	4.65	7.16		6.81	7.76	7.24	7.56	7.87	8.05	8.28
31	100		1.6–2.3	3.13	4.05		4.40	4.65	7.18		6.81	7.76	7.21	7.56	7.86	8.05	8.28
32	250		1.60–2.36	3.20	4.06		4.40	4.72	7.46		6.84	7.76	7.29	7.61	7.86	8.17	8.35
33	250		1.60–2.36	3.21	4.06		4.40	4.71	7.20		6.84	7.76	7.26	7.59	7.84	8.17	8.35

[a] Spectra determined in Me_2SO-d_6; the signals from the lactam and carboxyl hydrogens were not recorded. [b] n = 2–5. [c] n = 1–4.

Table V. Inhibition of Human (WI-L2) Thymidylate Synthase by 10-Propargyl-5,8-dideazafolic Acid and Its Polyglutamates

compd	K_i(app (nM) ± S.E.[a]	fold increase in inhibition
3, R = H	7.014 ± 0.488	
30	0.224 ± 0.034	31
31	0.072 ± 0.009	97
32	0.041 ± 0.008	171
33	0.042 ± 0.009	167

[a] K_i(app)'s were determined by using the Goldstein equation applicable to tight-binding inhibitors.[34]

tinued for 20 min at room temperature. *N*-Methylmorpholine hydrochloride was filtered off and the filtrate evaporated to dryness. The residue was dissolved in AcOEt and the resulting solution was washed with water and then brine. The dried (Na_2SO_4) extract was concentrated in vacuo to give the crude product, which was recrystallized from AcOEt–petrol. Yields and analytical data of products chromatographically homogeneous in system A are given in Table I.

Hydrogenolysis of 14, 15, 16, and 17 To Produce 18, 19, 20, and 21. A solution of the protected peptide **14**, **15**, **16**, or **17** (1 mmol) in MeOH (8 mL) containing Pd/C in suspension was stirred under hydrogen for 30 min to 1 h whereupon TLC (system B) showed the absence of starting material. The catalyst was removed by filtration and the filtrate evaporated to dryness. The resulting oil was used immediately in the next reaction.

Tri-*tert*-butyl *N*-[*N*-[4-(Prop-2-ynylamino)benzoyl]-L-γ-glutamyl]-L-glutamate (22) and Tetra-*tert*-butyl *N*-[*N*-[*N*-[4-(Prop-2-ynylamino)benzoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamate (23). A mixture of the appropriate primary amine **18** or **19** (10 mmol), propargyl bromide (10 mmol), and 2,6-dimethylpyridine (11 mmol) in DMF (15 mL) was stirred at room temperature (70 h for **18** and 120 h for **19**) with TLC monitoring in system C. The mixture was poured into water (120 mL) and extracted with AcOEt (6 × 60 mL), the combined extracts were washed sequentially with water and brine and dried (Na_2SO_4), and the solvent was removed in vacuo. The resulting oil was chromatographed on a silica gel column (95 × 5 cm) with AcOEt–CH_2Cl_2 (2:1) as the eluent. Concentration of the appropriate fractions gave an oil, which, triturated with petrol, went solid. The crude product **22** was recrystallized from ethyl acetate. Yields and analytical data of products chromatographically homogeneous in system C are given in Table II and ^{1}H NMR data in Table IV.

Penta-*tert*-butyl *N*-[*N*-[*N*-[*N*-[4-(Prop-2-ynylamino)benzoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamate (24) and Hexa-*tert*-butyl *N*-[*N*-[*N*-[*N*-[*N*-[4-(Prop-2-ynylamino)benzoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamate (25). A mixture of the appropriate primary amine **20** or **21** (2.5 mmol), $CaCO_3$ (2.5 mmol), and propargyl bromide (2.5 mmol) in DMA (2.5 mL) was stirred at room temperature for 60 h whereupon a slight impurity (the tertiary amine) appeared at higher R_f (TLC, system C). The solvent was removed in vacuo and ethyl acetate (5 mL) added. The inorganic salts were filtered off and the filtrate evaporated in vacuo to give an oil. This was chromatographed on a silica gel column (78 × 3 cm) with AcOEt–CH_2Cl_2 (2:1) as the eluent. Concentration of the appropriate fractions gave an oil that, triturated with petrol, went solid. The crude product **24** was recrystallized from ethyl acetate–petrol. Yields and analytical data of products chromatographically homogeneous in system C are given in Table II and ^{1}H NMR data in Table IV.

Tri-*tert*-butyl *N*-[*N*-[4-[*N*-[(2-Amino-4-hydroxy-6-quinazolinyl)methyl]prop-2-ynylamino]benzoyl]-L-γ-glutamyl]-L-glutamate (26), Tetra-*tert*-butyl *N*-[*N*-[*N*-[4-[*N*-[(2-Amino-4-hydroxy-6-quinazolinyl)methyl]prop-2-ynylamino]benzoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamate (27), Penta-*tert*-butyl *N*-[*N*-[*N*-[*N*-[4-[*N*-[(2-Amino-4-hydroxy-6-quinazolinyl)methyl]prop-2-ynylamino]benzoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamate (28), and Hexa-*tert*-butyl *N*-[*N*-[*N*-[*N*-[4-[*N*-[(2-Amino-4-hydroxy-6-quinazolinyl)-methyl]prop-2-ynylamino]benzoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamate (29). A

mixture of the appropriate propargylamine **22**, **23**, **24**, or **25**, 2-amino-6-(bromomethyl)-4-hydroxyquinazoline hydrobromide (1 molar equiv), and $CaCO_3$ (1 molar equiv) in DMA (2.5 mL per mmol) was stirred at room temperature for 132 h (**22**), 420 h (**23**), or 60 h (**24**, **25**) with TLC monitoring using $CHCl_3$–MeOH (10:1). The mixture was filtered and the solids washed with DMA and the combined filtrates were concentrated in vacuo. The resulting crude product, an oil, was purified by column chromatography on silica gel with $CHCl_3$–MeOH (10:1) as the eluent. Yields and analytical data of products chromatographically homogeneous in system G are given in Table II and ^1H NMR data in Table IV.

***N*-[*N*-[4-[*N*-[(2-Amino-4-hydroxy-6-quinazolinyl)methyl]prop-2-ynylamino]benzoyl]-L-γ-glutamyl]-L-glutamic Acid Trifluoroacetate Salt (30), *N*-[*N*-[*N*-[4-[*N*-[(2-Amino-4-hydroxy-6-quinazolinyl)methyl]prop-2-ynylamino]benzoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamic Acid Trifluoroacetate Salt (31), *N*-[*N*-[*N*-[*N*-[4-[*N*-[(2-Amino-4-hydroxy-6-quinazolinyl)methyl]prop-2-ynylamino]benzoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamic Acid Trifluoroacetate Salt (32), and *N*-[*N*-[*N*-[*N*-[4-[*N*-[(2-Amino-4-hydroxy-6-quinazolinyl)methyl]prop-2-ynylamino]benzoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamic Acid Trifluoroacetate Salt (33).** A solution of **26**, **27**, **28**, or **29** in TFA (10 mL per mmol) was kept for 1 h at room temperature whereupon TLC in system H showed the absence of starting material. The solution was added dropwise to diethyl ether (100 mL per mmol). The white solid that precipitated was purified by six cycles of centrifugation–decantation–resuspension in diethyl ether. The product was dried in vacuo over KOH at 65 °C overnight. Yields, analytical data, and HPLC data are given in Table III and ^1H NMR data in Table IV.

Biochemical Evaluation. Human thymidylate synthase was partially purified from a WI-L2 cell line that overproduces TS 200-fold owing to amplification of the TS gene.[36] The enzyme preparation and assay method were as previously described for L1210 TS[33,37] except that a much purer preparation of tetrahydrofolate was used (Fluka, Neu-Ulm, West Germany) and the assay contained 0.2% bovine serum albumin. Briefly, the 0.5-mL reaction mixture contained 25 nmol of [5-^{3}H]dUMP (40 μCi/μmol), 100 nmol of (±)-L-FH_4, 1 μmol of HCHO, 5 μmol of dithiothreitol, 0.05 mL of inhibitor, and 0.2 mL of enzyme preparation. CB3717 and its polyglutamate derivatives were dissolved in 0.15 M $NaHCO_3$ and then diluted to appropriate concentrations in H_2O. The reaction was started with the addition of enzyme (diluted to the appropriate activity in 0.125 M potassium phosphate buffer, pH 7.4, containing 3 mM dithiothreitol and 0.5% bovine serum albumin—the last named from Sigma Chemical Co., Poole, Dorset, UK.) The K_i apparent's were determined by using the Goldstein equation[34] applicable to tight-binding inhibitors (zone B kinetics).[38] The data was fitted to the equation by a non-linear least squares regression.[39]

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(36) O'Connor, B. M.; Jackman, A. L.; Crossley, P. H.; Calvert, A. H. *Brit. J. Cancer* **1988**, *58*, 237.
(37) Jackman, A. L.; Alison, D. L.; Calvert, A. H.; Harrap, K. R. *Cancer Res.* **1986**, *46*, 2810.
(38) Straus, O. H.; Goldstein, A. *J. Gen. Physiol.* **1943**, *26*, 559.
(39) Jennrich, R. J.; Sampson, P. F. *Technometrics* **1968**, *10*, 63.

Synthesis of an Analogue of Tabtoxinine as a Potential Inhibitor of D-Alanine:D-alanine Ligase (ADP Forming)

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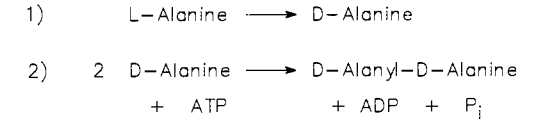
The design and synthesis of a potential inhibitor of D-alanine:D-alanine ligase (ADP forming) (EC 6.3.2.4) are described. This enzyme, which catalyzes the second step in the biosynthesis of bacterial peptidoglycan, is believed to generate D-alanyl phosphate as an enzyme-bound intermediate. With tabtoxinine, a potent inhibitor of glutamine synthetase, as a model, β-lactams **9R** and **9S** were synthesized as potential precursors of a D-alanyl phosphate mimic.

The dipeptide D-alanyl-D-alanine is known to be an essential precursor of bacterial peptidoglycan. Its synthesis in vivo (Scheme I) involves conversion of L-alanine to D-alanine by alanine racemase (EC 5.1.1.1), followed by coupling of two molecules of D-alanine by D-alanine:D-alanine ligase (ADP forming) (EC 6.3.2.4).[1] Inhibitors of alanine racemase have been extensively studied as potential antibacterial substances.[2] Inhibitors of the ligase include cycloserine (IC_{50} = 2.5 × 10^{-4} M)[3] and D-(1-aminoethyl)phosphonic acid (IC_{50} = 9.3 × 10^{-4} M).[4] Attempts to design more effective inhibitors of the ligase have accelerated. Inhibition of the ligase by several amino phosphonic and amino phosphonamidic acids has been described.[5] The synthesis and essentially irreversible

Scheme I. Biosynthesis of D-Alanyl-D-alanine from L-Alanine by the Action of the Enzymes (1) Alanine Racemase and (2) D-Alanine:D-alanine Ligase



(1) For a recent review, see: Ghuysen, J.-M. *Topics Antiobiot. Chem.* **1980**, *5*, 9.
(2) Neuhaus, F. C.; Hammes, W. P. *Pharmacol. Ther.* **1981**, *14*, 265.
(3) Neuhaus, F. C.; Lynch, J. L. *Biochemistry* **1964**, *3*, 471–480.
(4) Lacoste, A.-M.; Poulsen, M.; Cassigne, A.; Neuzil, E. *Curr. Microbiol.* **1979**, *2*, 113.

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