Non-glutamate Type Pyrrolo[2,3-*d*]pyrimidine Antifolates. I: Synthesis and Biological Properties of Pyrrolo[2,3-*d*]pyrimidine Antifolates Containing Tetrazole Congener of Glutamic Acid

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Either the α- or γ-carboxyl group of the glutamic acid moiety of *N*-[4-[3-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]benzoyl]-L-glutamic acid (1b, TNP-351) and its related compound (1a) was replaced with a 1*H*-tetrazole ring, and the inhibitory effects of the resulting compounds on dihydrofolate reductase (DHFR) and the growth of murine fibrosarcoma Meth A cells were examined. The γ-tetrazole analogs (2) were found to be much more potent DHFR inhibitors than TNP-351, and strongly inhibited the growth of Meth A cells. On the other hand, the α-tetrazole analogs (3) were much less active against Meth A cells, even though their DHFR-inhibitory activity was comparable to that of TNP-351. These findings suggest that the α-carboxyl group plays an important role in effective uptake *via* the reduced folate carrier, and a novel DHFR inhibitor could be obtained by chemically modifying the γ-carboxyl moiety while leaving the α-carboxyl group intact.

Key words non-glutamate type pyrrolo[2,3-*d*]pyrimidine antifolate; TNP-351; glutamic acid bioisostere; tetrazole containing antifolate; DHFR inhibition; antitumor activity

The antifolate methotrexate (MTX) has been used clinically for treating acute lymphocytic leukemia and choriocarcinoma for more than 30 years. Unfortunately, MTX is active against only a small group of solid tumors because of natural resistance, and continued therapy can cause acquired resistance in tumors that do respond initially. The main factors[1] known to be connected with resistance are (1) increased intracellular levels of dihydrofolate reductase (DHFR),[2] the enzyme whose inhibition is the primary mode of action of MTX, (2) impairment or loss of the reduced folate/MTX carrier (RFC) by which MTX is taken up into cells[3] and (3) decreased intracellular levels of folylpolyglutamate synthetase (FPGS),[4] the enzyme which converts MTX to its polyglutamates. These polyglutamates are more potent inhibitors of folate-requiring enzymes such as DHFR, thymidylate synthetase (TS), and aminoimidazolecarboxamide ribonucleotide transformylase (AICARTF), than MTX[5] and are not easily removed from the cells. Therefore, the glutamic acid moiety of MTX was thought to be necessary for biological activity. Recently, McGuire *et al.*[6] reported that γ-tetrazole analogs of MTX, in which the γ-carboxyl moiety of glutamic acid was replaced by a tetrazole ring, inhibited FPGS and were more efficiently transported into cells *via* the RFC and more active against human leukemia cells in culture than MTX. Barker *et al.*[7] reported that replacing the glutamic acid moiety of 2-methyl-N^{10}-propargyl-5,8-dideazafolic acid, a potent TS inhibitor, with amino acids containing an α-carboxyl group such as valine or *tert*-leucine did not affect TS inhibition and uptake *via* the RFC transport system. These findings suggest that modification of the glutamic acid moiety could lead to a novel type of antifolate.

We previously reported that *N*-[4-[3-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]benzoyl]-L-glutamic acid (**1b**, TNP-351), a novel DHFR inhibitor characterized by a pyrrolo[2,3-*d*]-pyrimidine ring, shows potent antitumor activities against not only leukemia cells but also solid tumor cells, both *in vitro*[8] and *in vivo*.[9] Our biological evaluations of TNP-351 demonstrated that it is more efficiently taken up into tumor cells and much more quickly converted to its polyglutamates than MTX,[9] and that these polyglutamates are potent inhibitors of AICARTF[10] as well as DHFR.[11]

As an extension of our research on TNP-351, we have modified its glutamic acid moiety and examined the biological activities of the modified compounds. Our first interest was the replacement of glutamic acid in TNP-351 with glutamic acid bioisosteres, in which each carboxyl group was replaced by a tetrazole ring, an isosteric group with acidic properties similar to a carboxyl group.

In the present report, we describe the preparation of pyrrolo[2,3-*d*]pyrimidine antifolates **2a**, **b** and **3a**, **b** with a glutamic acid bioisostere in place of glutamic acid. The inhibitory activities of these compounds against DHFR and the growth of murine fibrosarcoma Meth A cells are

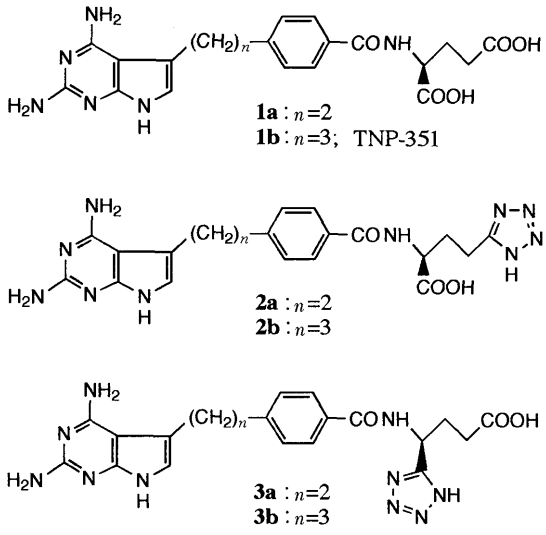

Fig. 1

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also reported.

Chemistry Pyrrolo[2,3-*d*]pyrimidinecarboxylic acids **4a, b** were synthesized by the method reported previously.[12] Pyrrolo[2,3-*d*]pyrimidine antifolates containing glutamic acid bioisosteres (**2a,b** or **3a, b**), in which one of the two carboxyl groups was replaced by a 1*H*-tetrazol-5-yl ring, were prepared by coupling **4a, b** and protected glutamic acid bioisosteres (**9**, **14**, **19**) followed by deprotection (Table I). The methods[7,13] reported previously for preparation of γ-tetrazolyl glutamic acid did not involve the protection of tetrazole. However, it seemed disadvantageous to use the nonprotected tetrazole compound compared with the protected one because of its high aqueous solubility, its high polarity and its tailing behavior on chromatography. Therefore, the synthesis of glutamic acid bioisosteres containing the γ-tetrazolyl group was carried out by two different procedures; the *N*-nonprotected tetrazole and *N*-protected tetrazole methods. The γ-nonprotected tetrazole compound **9** was prepared by modifying the procedure of Van *et al.*[13] The γ-carboxamide group of methyl *N*-benzyloxycarbonyl-L-glutaminate (**6**) was dehydrated to a nitrile (**7**) using benzenesulfonyl chloride in pyridine. The nitrile (**7**) thus obtained was reacted with trimethyltin azide to yield the tetrazole derivative **8**, from which the amino-protecting group Z was removed by catalytic hydrogenation over palladium carbon to give the γ-tetrazole analog **9** (Chart 1).

On the other hand, the γ-tetrazole compound (**14**) protected with a 2-cyanoethyl group was synthesized according to the method illustrated in Chart 2. The starting compound, α-methyl *N*-Boc-glutamate (**11**), was condensed with 3-aminopropionitrile in the presence of diethyl phosphorocyanidate (DEPC) to afford the *N*-Boc-protected methyl L-glutamic acid γ-cyanoethylamide (**12**), which was transformed to the cyanoethyl-protected tetrazole derivative **13** by the reaction of the amide moiety with trimethylsilyl azide in the presence of triphenylphos-

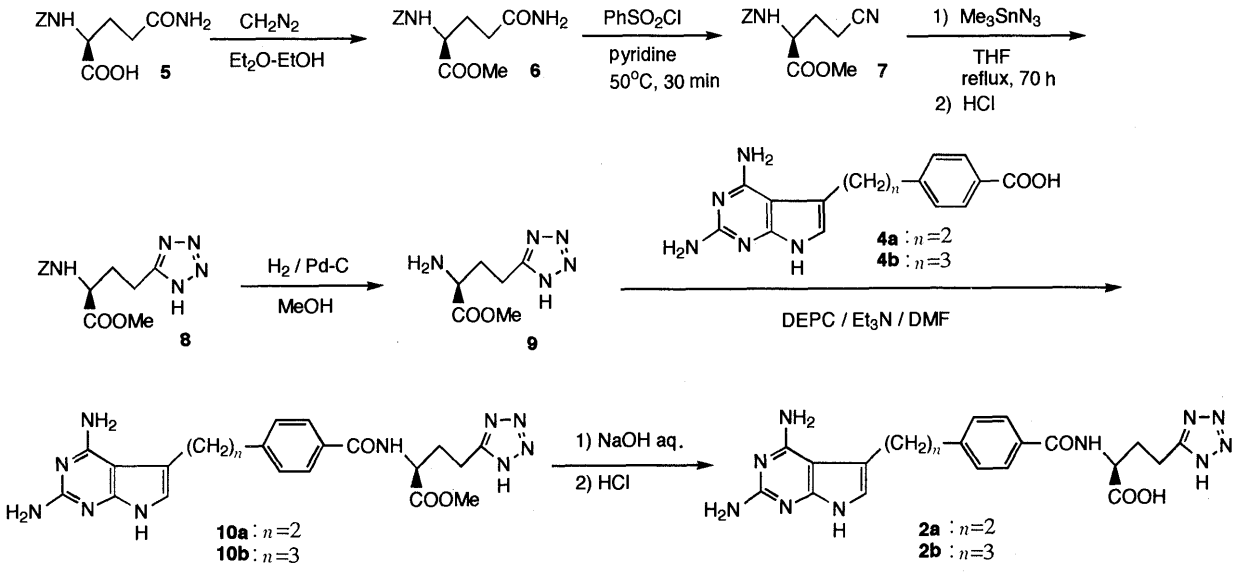


Chart 1

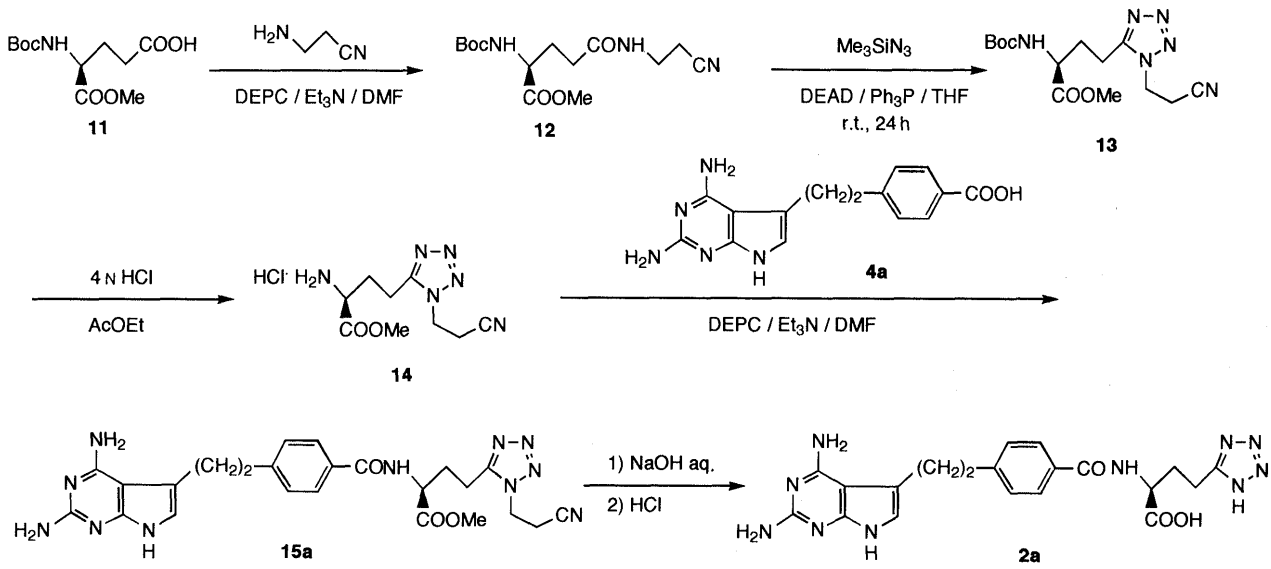


Chart 2

phine (Ph_3P) and diethyl azodicarboxylate (DEAD) in THF as reported by Duncia *et al.*[14] The glutamic acid bioisostere with a protected γ-tetrazole (**14**) was obtained by treating **13** with hydrochloric acid in AcOEt (Chart 2).

γ-Tetrazole analogs **9** and **14** prepared above were condensed with **4a** and **4b** in the presence of DEPC and triethylamine in DMF to obtain **10a, b** or **15a**. Finally **10a, b** and **15a** were treated with an aqueous solution of NaOH to remove the protecting groups, the methyl ester and cyanoethyl group at the 1 position on tetrazole, to afford **2a, b** (Charts 1 and 2). Comparison of the above two methods suggested that the tetrazole-protected method was superior to the nonprotected procedure with regard to purification of products and suitability for large-scale production. Therefore, we prepared the α-tetrazole-containing antifolates (**3a, 3b**) using the tetrazole-protected method as shown in Chart 3. The α-cyanoethylamide **17**, prepared from commercially available **16**, was converted to the protected tetrazole **18** *via* the key reaction with trimethylsilyl azide in the presence of Ph_3P and DEAD. After removal of the protecting Boc group, **19** was condensed with **4a** and **4b** to obtain **20a** and **20b**, which gave **3a** and **3b** upon deprotection (Chart 3).

Biological Activity and Discussion Inhibitory activities of the synthesized antifolates (**2a, b** and **3a, b**) against DHFR and the growth of murine fibrosarcoma Meth A cells were compared with those of TNP-351, the parent drug with an unmodified glutamic acid moiety (Table II). The inhibition of DHFR has been thought to be the primary mode of action of these tetrazole-containing compounds for two reasons: (1) they have two amino groups on the pyrimidine ring[15] like TNP-351 and MTX, which are DHFR inhibitors; and (2) they cannot be converted by FPGS to their polyglutamates, which could inhibit other folate-requiring enzymes in addition to DHFR.

The replacement of the α-carboxyl group on glutamic

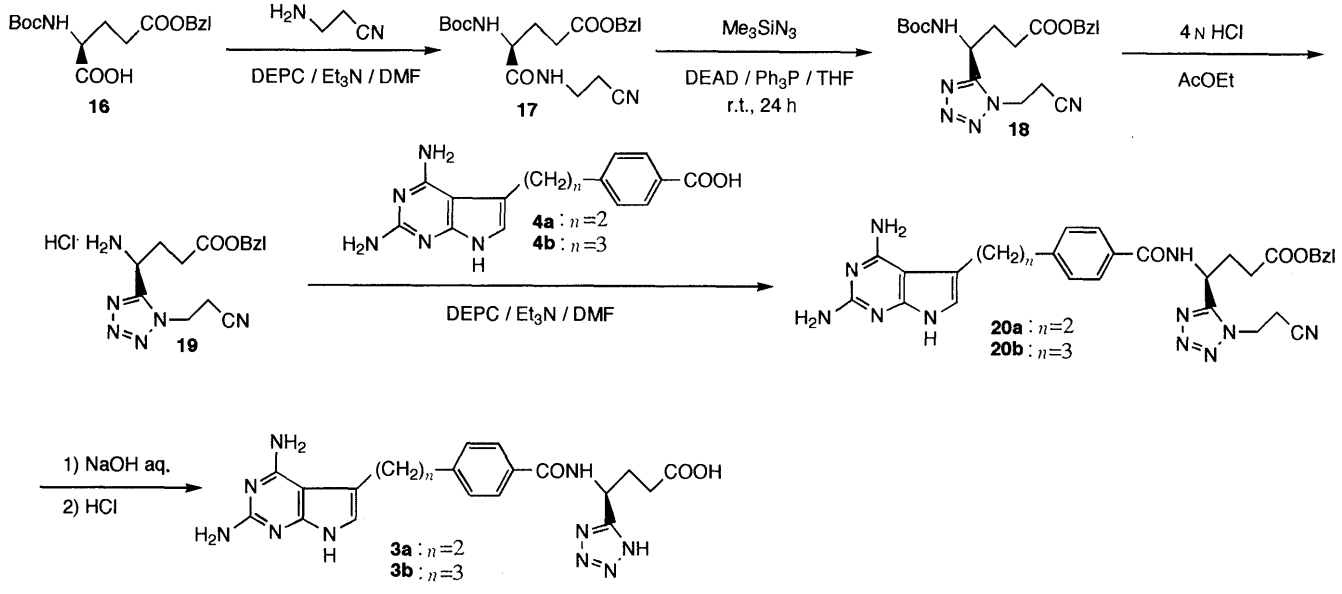
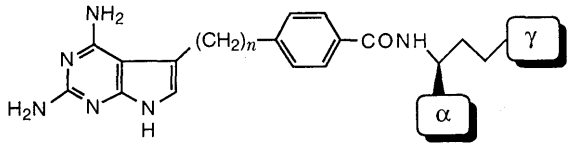


Chart 3

TABLE I. Preparation of Tetrazole-Containing Pyrrolo[2,3-*d*]pyrimidine Antifolates



Compd. No.	*n*	α	γ	Method[a]	Yield (%)	mp (°C)	HR-FABMS (M^+) Calcd	HR-FABMS (M^+) Found	Formula
17a	2	COOMe	Tet	A	70	201—203	465.2111[b]	465.2108[b]	$C_{21}H_{24}N_{10}O_3$
17b	3	COOMe	Tet	A	37	152—153	478.2189	478.2194	$C_{22}H_{26}N_{10}O_3$
19a	2	COOMe	Tet(CE)	A	25	119—120	517.2299	517.2286	$C_{24}H_{27}N_{11}O_3$
20a	2	Tet(CE)	COOBzl	A	41	105—106	593.2613	593.2600	$C_{30}H_{31}N_{11}O_3$
20b	3	Tet(CE)	COOBzl	A	54	88—89	607.2768	607.2775	$C_{31}H_{33}N_{11}O_3$
2a	2	COOH	Tet	B	65, 91[c]	200—202	450.1876	450.1851	$C_{20}H_{22}N_{10}O_3$
2b	3	COOH	Tet	B	70	174—175	464.2033	460.2039	$C_{21}H_{24}N_{10}O_3$
3a	2	Tet	COOH	B	85	195—196	450.1876	450.1870	$C_{20}H_{22}N_{10}O_3$
3b	3	Tet	COOH	B	85	178—180	464.2033	464.2034	$C_{21}H_{24}N_{10}O_3$

Tet, 1*H*-tetrazol-5-yl; Tet(CE), 1-(2-cyanoethyl)tetrazol-5-yl. *a*) A, DEPC/Et_3N; B, hydrolysis. *b*) (MH^+). *c*) This yield was obtained by hydrolysis of **19a**.

TABLE II. Inhibitory Effects of Tetrazole-Containing Pyrrolo[2,3-*d*]-pyrimidine Antifolates on DHFR and Meth A Cell Growth

Compd. No.	*n*	α	γ	IC_{50} (μM)	
				DHFR	Meth A
2a	2	COOH	Tet	0.0095	0.0013
2b	3	COOH	Tet	0.090	0.012
3a	2	Tet	COOH	0.36	0.61
3b	3	Tet	COOH	0.41	0.39
TNP-351 (**1b**)	3	COOH	COOH	0.37	0.00060

Tet: 1*H*-tetrazol-5-yl.

acid with a tetrazolyl group had little effect on the inhibitory activity against DHFR but resulted in a decrease in the inhibitory activity against the growth of Meth A cells. However, the conversion of the γ-carboxyl group to a tetrazolyl group led to an increase in DHFR-inhibitory activity and did not affect the potent inhibition of Meth A cell growth. With regard to the length of the spacer between the pyrrolo[2,3-*d*]pyrimidine ring and the benzene ring, in the case of the γ-tetrazole antifolate (**2**), the dimethylene analog **2a** showed more potent inhibition of both DHFR and Meth A cell growth than the trimethylene analog **2b**. These findings suggest that the α-carboxyl group plays an important role in effective cell entry *via* the RFC-mediated transport system, and that a γ-tetrazolyl group should be superior to a γ-carboxyl group for binding to DHFR. That is, the α-carboxyl group appears to be essential for efficient uptake into the cells *via* the RFC, and the γ-carboxyl region seems to play a major role in the binding to DHFR. In the case of pteridine antifolates such as MTX, it has been reported that replacement of the γ-carboxyl group with other groups such as tetrazole,[6] *tert*-butyl ester[16] and amides[17] does not greatly affect binding to DHFR. Therefore, our present results showing that the inhibitory activities of γ-tetrazole analogs **2a, b** against DHFR were much more potent than that of the γ-carboxyl analog (TNP-351: **1b**) are very interesting. However, the growth-inhibitory activities of the γ-tetrazole analogs **2a, b** were less potent than that of **1b**. This reversal may result from the longer retention of **1b** in the cells compared with **2a, b** and more potent DHFR inhibition by polyglutamates of **1b**, because **1b** is converted to its polyglutamates by intracelullar FPGS.

Taking into account the results described above, it may be possible to obtain novel DHFR inhibitors, which should be efficiently taken up into cells *via* the RFC and might also be active against MTX-resistant tumors with decreased FPGS level or impaired transport, by replacing the γ-carboxyl group with other appropriate groups, while leaving the α-carboxyl group intact.

Novel DHFR inhibitors based on the above approarch will be reported in a forthcoming paper.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. IR spectra were obtained on a JASCO IR-810 spectrometer. ^{1}H-NMR spectra were recorded on a Varian Gemini-200 spectrometer; chemical shifts are given in ppm with tetramethylsilane as the internal standard, and coupling constants (*J*) are given in hertz (Hz). Secondary ion mass spectra (SIMS) were determined on a Hitachi M-80B instrument. High-resolution mass spectra (HR-MS) and fast atom bombardment mass spectra (FAB-MS) were measured on a JEOL JMS-AX505W instrument. Column chromatography was carried out using Silica gel 60 (E. Merck, Darmstadt, Germany).

Methyl (*S*)-2-Amino-4-(1*H*-tetrazol-5-yl)butyrate (9) Methyl (*S*)-2-amino-4-cyanobutyrate (**7**) (5.8 g) was prepared from methyl *N*-benzyloxycarbonyl-L-glutaminate (**6**) (6.2 g) by a method similar to that reported by Gizonka *et al.*[13] A solution of **7** (2.76 g) and trimethyltinazide (2.06 g) in THF (16 ml) was refluxed for 72 h and cooled to room temperature. A 4 N HCl AcOEt solution was added to the reaction mixture, and the mixture was stirred for 1 h and then diluted with ether to give methyl (*S*)-2-benzyloxycarbonylamino-4-(1*H*-tetrazol-5-yl)butyrate (**8**) (1.02 g, 32%) as a white powder. IR (KBr): 3300, 1730, 1685, 1540, 1295, 1270 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.90—2.30 (2H, m), 2.96 (2H, t, *J*=7.7 Hz), 3.64 (3H, s), 4.14 (1H, m), 5.05 (2H, s), 7.36 (5H, s), 7.89 (1H, d, *J*=8.0 Hz). FAB-MS *m/z*: 319 (MH^+).

A solution of **8** and 10% Pd–C in methanol (20 ml) was hydrogenated overnight, filtered and concentrated *in vacuo* to give **9** (99%) as a white powder. mp: 158—160 °C. IR (KBr): 3400, 1740, 1615, 1580, 1505, 1270 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.90—2.25 (2H, m), 2.90—3.10 (2H, m), 3.44 (1H, t, *J*=6.0 Hz). FAB-MS *m/z*: 186 (MH^+).

***N*-(*tert*-Butyloxycarbonyl)-L-glutamic Acid α-(2-Cyanoethylamide) γ-Benzyl Ester (17)** Triethylamine (22.5 g) was added dropwise to a stirred solution of *N*-(*tert*-butyloxycarbonyl)-L-glutamic acid γ-benzyl ester (**16**, 16.0 g), 3-aminopropionitrile (3.35 g) and DEPC (11.5 g) in dry DMF (20 ml) under ice cooling. The reaction mixture was stirred for 30 min at room temperature and then concentrated *in vacuo*, and the residue was dissolved in AcOEt (100 ml). The AcOEt solution was successively washed with water and brine, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was then chromatographed on silica gel (AcOEt–hexane–$CHCl_3$, 2:4:1) to give **17** (17.67 g, 96%) as colorless needles. mp: 95—96 °C. IR (KBr): 3330, 1720, 1680, 1655, 1525, 1295, 1160 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.44 (9H, s), 1.85—2.25 (2H, m), 2.40—2.65 (4H, m), 3.50 (2H, q, *J*=6.4 Hz), 4.16 (1H, m), 5.13 (2H, s), 5.30 (1H, br d, *J*=7.8 Hz), 6.89 (1H, br t, *J*=6.0 Hz), 7.36 (5H, s). SIMS *m/z*: 390 (MH^+).

Benzyl (*S*)-4-Amino-4-[1-(cyanoethyl)tetrazol-5-yl]butyrate Hydrochloride (19) Diethyl azodicarboxylate (0.95 ml) was added dropwise to a stirred solution of **17** (1.17 g), triphenylphosphine (1.57 g) and trimethylsilyl azide (0.80 ml) in dry THF (20 ml) at 0 °C in an argon atmosphere. The mixture was stirred at room temperature for 24 h, treated with an aqueous solution of cerium(IV) ammonium nitrate and diluted with dichloromethane (100 ml). The organic layer was successively washed with water and brine, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was then chromatographed on silica gel (AcOEt–hexane–$CHCl_3$, 2:6:1) to give benzyl (*S*)-4-(*tert*-butyloxycarbonylamino)-4-[1-(cyanoethyl)tetrazol-5-yl]butyrate (**18**). A 4 N HCl AcOEt solution (5 ml) was added to solution of **18** in AcOEt (5 ml). The reaction mixture was stirred for 30 min and concentrated to obtain **19** (740 mg, 70%) as a colorless wax. IR (KBr): 3500—2500, 1720, 1680, 1525, 1430, 1250, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.40—2.75 (4H, m), 3.11 (2H, t, *J*=6.4 Hz), 4.60—4.90 (2H, m), 4.99 (2H, s), 5.38 (1H, m), 7.28 (5H, s), 9.05 (3H, br s). SIMS *m/z*: 315 (MH^+).

Methyl N^{α}-(*tert*-Butyloxycarbonyl)-*N*-(2-cyanoethyl)-L-glutaminate (12) Compound **12** (1.77 g, 74%) was synthesized from *N*-*tert*-butyloxycarbonyl-L-glutamic acid α-methyl (2.0 g), 3-aminopropionitrile (640 mg), DEPC (1.87 g) and triethylamine (3.10 g) by the same method as that described for **17**. Colorless solid. mp: 104—105 °C. IR (KBr): 3350, 3310, 2245, 1750, 1740, 1680, 1650, 1535, 1520, 1440, 1365, 1340, 1320, 1280, 1260, 1220, 1170 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.45 (9H, s), 1.80—2.00 (1H, m), 2.10—2.40 (3H, m), 2.64 (2H, t, *J*=6.4 Hz), 3.52 (2H, q, *J*=6.4 Hz), 3.75 (3H, s), 4.30 (1H, m), 5.30 (1H, d, *J*=7.8 Hz), 6.81 (1H, br s). SIMS *m/z*: 314 (MH^+).

Methyl (*S*)-2-(*tert*-Butyloxycarbonylamino)-4-[1-(2-cyanoethyl)tetrazol-5-yl]butyrate (13) Compound **13** (1.53 g, 82%) was synthesized from **12** (1.726 g) in the presence of triphenylphosphine (4.32 g), trimethylsilyl azide (2.2 ml) and DEAD (2.61 ml) by the same method as that described for **18**. Pale yellow oil. IR (neat): 3350, 3245, 3050, 2975, 2250, 1735, 1705, 1690, 1520, 1505, 1435, 1390, 1360, 1170, 1115, 720, 690, 540 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.44 (9H, s), 2.24 (1H, m), 2.58 (1H, m), 3.01 (2H, t, *J*=7.4 Hz), 3.09 (2H, t, *J*=6.8 Hz), 3.75 (3H, s), 4.32 (1H, dt, *J*=8.2, 4.6 Hz), 4.56 (2H, t, *J*=6.8 Hz), 5.30 (1H, br d, *J*=7.0 Hz). MS *m/z*: 282 (MH^+ − *tert*-Bu), 279 (M^+ − COOMe), 237 (M^+ − COO*tert*-Bu), 223

($MH^+ - COOMe - tert\text{-}Bu$), 179 ($MH^+ - COOMe - COOtert\text{-}Bu$).

Methyl (*S*)-2-Amino-4-[1-(2-cyanoethyl)tetrazol-5-yl]butyrate Hydrochloride (14) A 4 N HCl AcOEt solution (10 ml) was added to a stirred solution of **13** (1.52 g) in AcOEt (4 ml). The reaction mixture was stirred for 10 min and concentrated *in vacuo* to give **14** (1.07 g, 100%) as a pale yellow wax. IR (neat): 3370, 3300, 2950, 2245, 1730, 1515, 1450, 1430, 1410, 1200, 1110 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 2.11 (1H, m), 2.40 (1H, m), 3.10 (2H, t, J=7.6 Hz), 3.11 (2H, t, J=6.6 Hz), 3.49 (1H, m), 3.71 (3H, s), 4.65 (2H, t, J=6.6 Hz). MS *m/z*: 179 ($M^+ - COOMe$).

Methyl (*S*)-2-[4-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]amino-4-(1*H*-tetrazol-5-yl)butyrate (10a, Table I) Triethylamine (500 mg) was added dropwise to a stirred solution of **9** (75 mg), 4-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoic acid ditrifluoroacetate (**4a**) (210 mg) and DEPC (100 mg) in dry DMF (6 ml) under ice cooling. The reaction mixture was stirred for 4 h at room temperature and then concentrated *in vacuo*, and the residue was dissolved in AcOEt (100 ml). The AcOEt solution was successively washed with water and brine, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was then chromatographed on silica gel ($CHCl_3$–methanol–H_2O, 100 : 25 : 4) to give **10a** (130 mg, 70%) as a colorless solid. IR (KBr): 3340, 3200, 1730, 1640, 1540, 1500, 1435, 1220, 1090, 1055 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 2.10—2.40 (2H, m), 2.97 (6H, m), 3.65 (3H, s), 4.51 (1H, m), 5.45 (2H, br s), 6.07 (2H, br s), 6.38 (1H, s), 7.35 (2H, d, J=7.8 Hz), 7.84 (2H, d, J=7.8 Hz), 9.09 (1H, d, J=6.4 Hz), 10.42 (1H, br s). FAB-MS *m/z*: 465 (MH^+).

Methyl (*S*)-2-[4-[3-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)-propyl]benzoyl]amino-4-(1*H*-tetrazol-5-yl)butyrate (10b, Table I) Compound **10b** (76 mg, 37%) was synthesized from **9** (80 mg) and 4-[3-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]benzoic acid (**4b**) (135 mg) by the same method as that described for **10a**. Colorless solid. IR (KBr): 3350, 3200, 2940, 1740, 1645, 1545, 1500, 1440, 1400, 1220, 1060, 770 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 1.70—1.95 (2H, m), 2.15—2.35 (2H, m), 2.60—2.80 (4H, m), 2.95 (2H, t, J=7.4 Hz), 3.65 (3H, s), 4.47 (1H, m), 5.48 (2H, br s), 6.08 (2H, br s), 6.44 (1H, s), 7.31 (2H, d, J=8.0 Hz), 7.84 (2H, d, J=8.0 Hz), 9.04 (1H, d, J=7.2 Hz), 10.47 (1H, br s). FAB-MS *m/z*: 478 (M^+), 479 (MH^+).

(*S*)-2-[4-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-benzoyl]amino-4-(1*H*-tetrazol-5-yl)butyric Acid (2a, Table I) A 1 N NaOH aqueous solution (2 ml) was added to a stirred solution of **10a** (115 mg) in methanol–THF (2 : 1; 6 ml) at room temperature. The reaction mixture was stirred for 1 h, concentrated *in vacuo* and dissolved in water, and then 1 N HCl (2 ml) was added to the mixture. The resulting precipitate was collected, washed with water and dried *in vacuo* to give **2a** (72 mg, 65%) as a white powder. Colorless solid. IR (KBr): 3340, 3200, 2920, 1650, 1545, 1500, 1460, 1390, 1290, 1250, 1090, 1060 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 2.10—2.45 (2H, m), 2.85—3.05 (6H, m), 4.43 (1H, m), 5.94 (2H, br s), 6.44 (1H, s), 6.58 (2H, br s), 7.33 (2H, d, J=8.2 Hz), 7.81 (2H, d, J=8.2 Hz), 8.65 (1H, d, J=7.8 Hz), 10.70 (1H, br s). FAB-MS *m/z*: 450 (M^+), 451 (MH^+).

(*S*)-2-[4-[3-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]-benzoyl]amino-4-(1*H*-tetrazol-5-yl)butyric Acid (2b, Table I) Compound **2b** (48 mg, 70%) was synthesized from **10b** (70 mg) by the same method as that described for **2a**. Colorless solid. IR (KBr): 3325, 3190, 2920, 1640, 1540, 1495, 1450, 1380, 1300, 1280, 1240, 760 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 1.75—1.95 (2H, m), 2.10—2.45 (2H, m), 2.60—2.80 (4H, m), 2.99 (2H, t, J=7.6 Hz), 4.40 (1H, m), 6.20 (2H, br s), 6.56 (1H, s), 6.82 (2H, s), 7.31 (2H, d, J=8.0 Hz), 7.82 (2H, d, J=8.0 Hz), 8.64 (1H, d, J=7.6 Hz), 10.91 (1H, br s). FAB-MS *m/z*: 464 (M^+), 465 (MH^+).

Benzyl (*S*)-4-[1-(Cyanoethyl)tetrazol-5-yl]-4-[4-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]aminobutyrate (20a, Table I) Triethylamine (500 mg) was added dropwise to a stirred solution of **19** (300 mg), **4a** (300 mg) and DEPC (150 mg) in dry DMF (10 ml) under ice cooling. The reaction mixture was stirred for 30 min at room temperature and then concentrated *in vacuo*, and the residue was dissolved in AcOEt (100 ml). The AcOEt solution was successively washed with water and brine, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was then chromatographed on silica gel ($CHCl_3$–methanol, 15 : 1 → 10 : 1) to give **20a** (138 mg, 41%) as a colorless solid. IR (KBr): 3380, 2255, 1730, 1610, 1580, 1550, 1500, 1460, 1430, 1170, 750 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 2.20—2.80 (4H, m), 2.95 (4H, br s), 3.23 (2H, t, J=6.0 Hz), 4.77 (2H, t, J=6.6 Hz), 5.08 (2H, s), 5.37 (2H, br s), 5.51 (1H, m), 5.97 (2H, br s), 6.36 (1H, s), 7.33 (2H, d, J=8.0 Hz), 7.36 (5H, s), 7.79 (2H, d, J=8.0 Hz), 9.10 (1H, d, J=7.4 Hz), 10.36 (1H, br s). FAB-MS *m/z*: 593 (M^+), 594 (MH^+).

Benzyl (*S*)-4-[1-(Cyanoethyl)tetrazol-5-yl]-4-[4-[3-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]benzoyl]aminobutyrate (20b, Table I) Compound **20b** (319 mg, 54%) was synthesized from **19** (508 mg) and **4b** (300 mg) by the same method as that described for **20a**. Colorless solid. IR (KBr): 3470, 3360, 3180, 2930, 2245, 1725, 1605, 1570, 1540, 1490, 1450, 1420, 1320, 1290, 1160, 750 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 1.83 (2H, m), 2.30—2.80 (8H, m), 3.22 (2H, t, J=6.6 Hz), 4.76 (2H, t, J=6.6 Hz), 5.07 (2H, s), 5.33 (2H, br s), 5.50 (1H, m), 5.89 (2H, br s), 6.41 (1H, s), 7.29 (2H, d, J=8.2 Hz), 7.34 (5H, s), 7.80 (2H, d, J=8.2 Hz), 9.09 (1H, d, J=7.8 Hz), 10.36 (1H, br s). FAB-MS *m/z*: 607 (M^+), 608 (MH^+).

(*S*)-4-[4-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-benzoyl]amino-4-(1*H*-tetrazol-5-yl)butyric Acid (3a, Table I) A 1 N NaOH aqueous solution (2 ml) was added to a stirred solution of **20a** (123 mg) in methanol–THF (2 : 1; 6 ml) at room temperature. The reaction mixture was stirred for 1 h, and concentrated *in vacuo*. The residue was dissolved in water, and then 1 N HCl (2 ml) was added. The resulting precipitate was collected, washed with water and dried *in vacuo* to give **3a** (80 mg, 85%) as a white solid. IR (KBr): 3330, 3200, 2920, 1650, 1540, 1460, 1385, 1190, 1085 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 2.10—2.70 (4H, m), 2.97 (4H, br s), 5.42 (1H, m), 5.84 (2H, br s), 6.44 (1H, s), 6.48 (2H, br s), 7.34 (2H, d, J=8.0 Hz), 7.82 (2H, d, J=8.0 Hz), 8.88 (1H, d, J=7.2 Hz), 10.66 (1H, br s). FAB-MS *m/z*: 450 (M^+), 451 (MH^+).

(*S*)-4-[4-[3-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]-benzoyl]amino-4-(1*H*-tetrazol-5-yl)butyric Acid (3b, Table I) Compound **3b** (170 mg, 85%) was synthesized from **20b** (262 mg) by the same method as that described for **3a**. Colorless solid. IR (KBr): 3340, 3175, 2930, 1645, 1540, 1500, 1460, 1390, 1330, 1300, 1255, 1190, 820, 770 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 1.75—1.95 (2H, m), 2.10—2.45 (4H, m), 2.68 (4H, br s), 5.41 (1H, m), 5.99 (2H, br s), 6.53 (1H, s), 6.58 (2H, br s), 7.31 (2H, d, J=8.0 Hz), 7.83 (2H, d, J=8.0 Hz), 8.89 (1H, d, J=8.0 Hz), 10.78 (1H, br s). FAB-MS *m/z*: 464 (M^+), 465 (MH^+).

Methyl (*S*)-4-[1-(Cyanoethyl)tetrazol-5-yl]-2-[4-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]aminobutyrate (15a, Table I) Compound **15a** (306 mg, 25%) was synthesized from **14** (1.09 g) and **4a** (1.20 g) by the same method as that described for **20a**. Colorless solid. IR (KBr): 3340, 3210, 2250, 1730, 1655, 1620, 1575, 1540, 1500, 1490, 1445, 1415, 1380, 1310, 1245 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 2.20—2.45 (2H, m), 2.90—3.10 (6H, m), 3.17 (2H, t, J=6.6 Hz), 3.67 (3H, s), 4.64 (1H, m), 4.67 (2H, t, J=6.6 Hz), 5.35 (2H, s), 5.97 (2H, br s), 6.37 (1H, s), 7.35 (2H, d, J=8.4 Hz), 7.81 (2H, d, J=8.4 Hz), 8.78 (1H, d, J=7.2 Hz), 10.35 (1H, br s). FAB-MS *m/z*: 517 (M^+), 518 (MH^+).

Compound 2a, Table I Compound **2a** (232 mg, 91%) was also synthesized from **15a** (294 mg) by the same method as that described for **3a**.

Cell Growth Inhibition Assay Meth A cells were grown in MEM (minimum essential medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 at 37 °C. Logarithmically growing cells (4×10^4) in 2.0 ml of medium were seeded in 12-well plates. Test drugs were added at various concentrations prior to the cell seeding. Cells were incubated for 72 h, and the cell number was counted with a Coulter Counter, model ZM (Coulter Electronics Ltd., Luton, England).

DHFR Inhibition Assay DHFR activity was measured by optical photometry using a modification of the method of Bertino *et al.*[18] The reaction was carried out at 30 °C in flat-bottomed 96-well plates (Nunc-Immunoplate Maxisorp) pretreated with 1 mg/ml bovine serum albumin utilizing 0.25 μg protein/ml (1.9 μU/ml) bovine liver DHFR, various concentrations of dihydrofolic acid and 300 μl of a reaction buffer containing 0.1 M Tris–HCl (pH 7.5), 150 mM KCl, 15 mM 2-mercaptoethanol and 125 mM NADPH. The reaction was started by adding a mixture of NADPH and dihydrofolic acid to the reaction buffer containing DHFR and drugs. Changes in the absorbance of NADPH and dihydrofolic acid were measured at 340 nm using a Titertek Multiskan MCC/340 (Labsystems, Finland) controlled by a personal computer for 2 min at 2 s intervals.

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References

1) J. R. Bertino, S. Srimatkandada, M. D. Carman, M. Jastreboff, L. Mehlman, W. D. Medina, E. Mini, B. A. Moroson, A. R. Cashmore, S. K. Dube, "New Experimental Modalities in the Control of

Neoplasia," ed. by P. Chandra, Plenum Publishing Corp., New York, 1986, pp. 183—193.
2) S. Srimatkandada, W. D. Medina, A. R. Cashmore, W. Whyte, D. Engel, B. A. Moroson, C. T. Franco, S. K. Dube, J. R. Bertino, *Biochemistry*, **22**, 5774 (1983).
3) G. A. Fischer, *Biochem. Pharmacol.*, **11**, 1233 (1962).
4) K. H. Cowan, J. A. Jolivet, *J. Biol. Chem.*, **259**, 10793 (1984); G. Pizzorno, E. Mini, M. Coronnello, J. J. McGuire, B. A. Moroson, A. R. Cashmore, R. N. Dreyer, J. T. Lin, T. Mazzei, P. Periti, J. R. Bertino, *Cancer Res.*, **48**, 2149 (1988).
5) C. J. Allegra, B. A. Chabner, J. C. Lutz, D. Rodbard, J. Jolivet, *J. Biol. Chem.*, **260**, 9720 (1985); C. J. Allegra, J. C. Drake, J. Jolivet, B. A. Chabner, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 4881 (1985).
6) J. J. McGuire, C. A. Russell, W. E. Bolanowska, C. M. Freitag, C. S. Jones, T. I. Kalman, *Cancer Res.*, **50**, 1726 (1990).
7) A. J. Barker, F. T. Boyle, A. L. Jackman, A. H. Calvert, S. J. Pegg, T. C. Stephens, L. R. Hughes, *Proc. Am. Assoc. Cancer Res.*, **32**, 327 (1991).
8) T. Miwa, H. Hitaka, H. Akimoto, H. Nomura, *J. Med. Chem.*, **34**, 555 (1991).
9) H. Akimoto, T. Hitaka, T. Miwa, K. Yukishige, T. Kusanagi, K. Ootsu, *Proc. Am. Assoc. Cancer Res.*, **32**, 327 (1991).
10) F. Itoh, O. Russello, H. Akimoto, G. P. Beardsley, *Cancer Chemother. Pharmacol.*, **34**, 273 (1994).
11) K. Yukishige, T. Kusanagi, T. Fujita, K. Aso, H. Akimoto, K. Ootsu, *Proc. Jpn. Cancer Assoc.*, **1991**, 385.
12) T. Miwa, T. Hitaka, H. Akimoto, *J. Org. Chem.*, **58**, 1696 (1993).
13) T. T. Van, E. Kojro, Z. Gizonka, *Tetrahedron*, **33**, 2299 (1977).
14) J. V. Duncia, M. E. Pierce, J. B. Santella III, *J. Org. Chem.*, **56**, 2395 (1991).
15) A. Rosowsky, *Prog. Med. Chem.*, **26**, 1 (1989).
16) A. Rosowsky, R. Forsch, J. Uren, M. Wick, *J. Med. Chem.*, **24**, 1450 (1981).
17) J. R. Piper, J. A. Montgomery, F. M. Sirotnak, P. L. Chello, *J. Med. Chem.*, **25**, 182 (1982); A. Rosowsky, H. Bader, M. R. Smith, C. A. Cucchi, M. Wick, J. H. Freisheim, *ibid.*, **29**, 1703 (1986); A. Rosowsky, H. Bader, C. A. Cucchi, R. G. Moran, W. Korler, J. H. Freisheim, *ibid.*, **31**, 1332 (1988); A. Rosowsky, H. Bader, J. H. Freisheim, *ibid.*, **34**, 574 (1991).
18) J. R. Bertino, J. P. Perkins, D. G. Johns, *J. Biol. Chem.*, **248**, 984 (1973).