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Synthesis and Antitumor Activity of Pyrrolo[2,3-*d*]pyrimidine Antifolates with a Bridge Chain Containing a Nitrogen Atom

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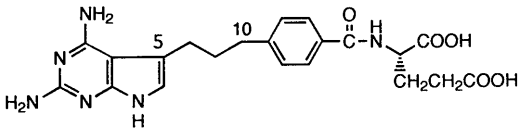
Novel pyrrolo[2,3-*d*]pyrimidine antifolates (1a, b and 2a, b) with a nitrogen atom in the bridge chain between the 2,4-diaminopyrrolo[2,3-*d*]pyrimidine and phenylene rings were designed and efficiently synthesized. These compounds exhibited more potent inhibitory activities than methotrexate (MTX) against the proliferation of human epidermoid carcinoma KB cells and human non-small cell lung carcinoma A549 cells despite their modest dihydrofolate reductase (DHFR)-inhibitory potency.

Key words pyrrolo[2,3-*d*]pyrimidine; antifolate; dihydrofolate reductase; antitumor activity

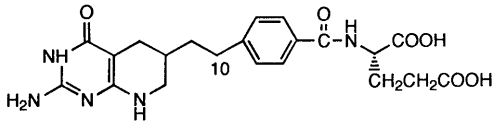
Methotrexate (MTX) is a well-known chemotherapeutic agent for the treatment of acute leukemia, chronic lymphocytic leukemia, chronic myelocytic leukemia, and choriocarcinoma.[1] MTX exerts its major therapeutic effects by inhibiting dihydrofolate reductase (DHFR), which is one of the most important enzymes involved in the biosynthesis of nucleic acids. Since the initial use of MTX in 1953, vigorous research have been conducted to discover novel folate analogues without some of the drawbacks of MTX,[2] *i.e.* low chemotherapeutic index against most human solid tumors, severe toxicity to normal tissues, especially in the gastrointestinal tract, and emergence of drug resistance following clinical use,[3] but so far without success.

Recently, we synthesized novel antifolates characterized by a structurally unique pyrrolo[2,3-*d*]pyrimidine ring with an alkylene bridge at the C-5 position.[4–6] Among them, *N*-[4-[3-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]benzoyl]-L-glutamic acid (TNP-351) exhibited potent growth-inhibitory activities against some solid tumors (mouse fibrosarcoma Meth A, mouse Lewis lung carcinoma 3LL, mouse colon carcinoma 26) and MTX-resistant mouse lymphocytic leukemia P388 *in vivo* as well as human epidermoid carcinoma KB cells and non-small cell lung carcinoma A549 cells *in vitro*.[7] The antitumor activity of TNP-351 was shown to be more potent than that of MTX and was antagonized by leucovorin, both *in vitro* and *in vivo*, indicating inhibitory potency against DHFR.[8] The DHFR-inhibitory potency of TNP-351 was, however, not as strong as that of MTX. It was demonstrated that the modest DHFR-inhibitory potency was well compensated for by good uptake of TNP-351 by tumor cells and its excellent substrate activity for folylpolyglutamate synthase (FPGS).[9] Therefore, enhancement of DHFR-inhibitory potency was expected to result in further improvement of the antitumor activity of TNP-351. Introduction of a nitrogen into the bridge chain of TNP-351, as seen in natural folic acid and potent DHFR inhibitors such as MTX and aminopterin, seemed to be a rational modification to elevate the DHFR-inhibitory potency and increase the antitumor activity. Recently, Taylor and co-workers reported that the substitution of a nitrogen at the 10-carbon atom of 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF) enhanced the growth-inhibitory activity of DDATHF in cultured leukemia cells.[10]

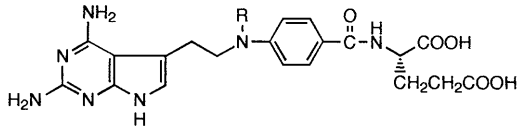
Taking these results into consideration, we introduced a nitrogen atom into the bridge chain of TNP-351. In this report, we describe an efficient method for the synthesis of new pyrrolo[2,3-*d*]pyrimidine antifolates (**1a, b** and **2a, b**) with a nitrogen atom (–NMe–, –NH–) at the C-10 position, together with their DHFR-inhibitory potency and their growth-inhibitory activities against KB cells and



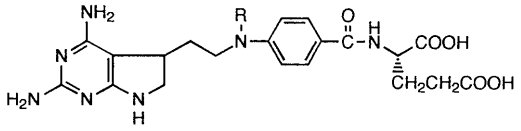
TNP-351



DDATHF



1a: R = CH_3
2a: R = H



1b: R = CH_3
2b: R = H

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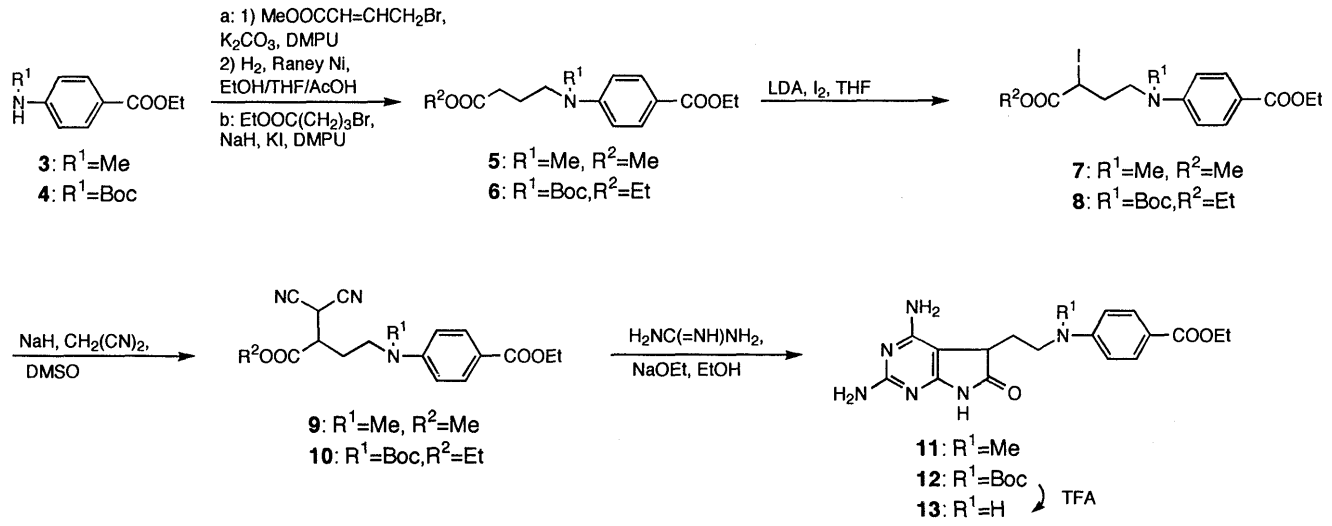

3: R^1=Me
4: R^1=Boc
5: R^1=Me, R^2=Me
6: R^1=Boc,R^2=Et
7: R^1=Me, R^2=Me
8: R^1=Boc,R^2=Et
9: R^1=Me, R^2=Me
10: R^1=Boc,R^2=Et
11: R^1=Me
12: R^1=Boc
13: R^1=H

Chart 1

A549 cells *in vitro*.

Chemistry Our synthetic strategy applied to the preparation of new pyrrolo[2,3-*d*]pyrimidine antifolates (**1a**, **b** and **2a**, **b**) involves one-step cyclization of α-dicyanomethylbutylate derivatives (**9** and **10**) with guanidine to 6-oxopyrrolo[2,3-*d*]pyrimidines (**11** and **12**) as the key reaction, as used in our previous study.[5] The α-dicyanomethylbutyrates (**9** and **10**) were derived from 4-aminobenzoic acid derivatives (**3** and **4**) *via* the steps shown in Chart 1. Ethyl 4-methylaminobenzoate (**3**) was coupled with methyl 4-bromocrotonate in the presence of K_2CO_3 in 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (DMPU)[11] at room temperature and the resulting 4-aminocrotonate was hydrogenated over Raney Ni to give the 4-aminobutyrate derivative (**5**). Ethyl 4-(*tert*-butoxycarbonylamino)benzoate (**4**) prepared from ethyl 4-aminobenzoate was directly alkylated with ethyl 4-bromobutyrate in DMPU in the presence of NaH and NaI to afford the 4-aminobutyrate derivative (**6**). Iodination of the 4-aminobutyrates (**5** and **6**) with lithium diisopropylamide (LDA) and I_2 in tetrahydrofuran (THF) at −78 °C,[12] and subsequent treatment with the sodium salt of malononitrile in dimethyl sulfoxide (DMSO) at room temperature afforded the key intermediary α-dicyanomethylbutyrate derivatives (**9** and **10**). Formation of the pyrrolo[2,3-*d*]pyrimidine ring, a key step in our strategy, was achieved by reacting the α-dicyano compounds (**9** and **10**) with guanidine hydrochloride in the presence of EtONa in refluxing EtOH, affording the 6-oxopyrrolo[2,3-*d*]pyrimidine derivatives, **11** and **12**, in 74% and 85% yields, respectively. Deprotection of **12** with trifluoroacetic acid (TFA) gave **13** (Chart 1).

For the reduction of the lactam carbonyl at the C-6 position of **11**, the use of BH_3–THF complex was most effective.[13] Treatment of **11** with BH_3–THF complex in THF at 0 °C, followed by decomposition of the borane–product complex with AcOH–EtOH, gave a mixture of pyrrolo[2,3-*d*]pyrimidine **14a** and its dihydro derivative **14b**, which were easily separated by flash chromatography. However, a similar reduction of **13** resulted in concomitant formation of the tricyclic compound (**17**) in addition to the desired product (**18**). The formation of **17** was assumed to be a result of intramolecular attack on the C-6 carbon of the intermediate **16** by the secondary amine on the bridge chain (path A). On the other hand, the attack of a hydride anion on the C-6 carbon gave **18** (path B). The structure of **17** was determined on the basis of its ^{1}H-NMR spectrum. The most diagnostic feature of the ^{1}H-NMR spectrum was a doublet appearing at 5.62 ppm, attributable to the methine proton at the C-7a position. This compound **17** could be easily converted into the desired bicyclic compound (**19a**) by alkaline treatment. Hydrolysis of the ester groups of **14a**, **b** and **18** with 1 N aqueous NaOH provided the corresponding carboxylic acids (**15a**, **b** and **19b**). The acids (**15a**, **b** and **19a**, **b**) were used in the following reaction without purification (Chart 2).

The benzoic acid derivatives (**15a**, **b** and **19a**, **b**) were coupled with diethyl L-glutamate *via* the acyl azides generated *in situ* by diphenylphosphoryl azide (DPPA)[14] to yield the diethyl esters (**20a**, **b** and **21a**, **b**). Finally, hydrolysis of the **20a**, **b** and **21a**, **b** with 1 N aqueous NaOH gave the desired antifolates (**1a**, **b** and **2a**, **b**) (Chart 3). The ^{1}H-NMR spectra of the N^{10}-methyl antifolate (**1b**) and N^{10}-unsubstituted glutamate (**21b**) indicated that they were mixtures of two diastereomers (*d*L and *l*L). Thus, the N^{10}-methyl glutamate (**20b**) was deduced to be generated as a diastereomeric mixture. The N^{10}-unsubstituted antifolate (**2b**) was also presumed to be a diastereomeric mixture, although the existence of diastereomers was not detected by ^{1}H-NMR. The physicochemical properties of the antifolates thus obtained (**1a**, **b** and **2a**, **b**) are summarized in Table I.

Biological Results and Discussion

The pyrrolo[2,3-*d*]pyrimidine antifolates **1a**, **b** and **2a**, **b** synthesized as above were examined for their inhibitory effects on bovine liver DHFR. They were also tested for their growth-inhibitory activities against KB cells and A549 cells *in vitro*. These results were compared with those for TNP-351 and MTX (Table II). Compounds **1b** and **2b** were evaluated as mixtures of two diastereomers.

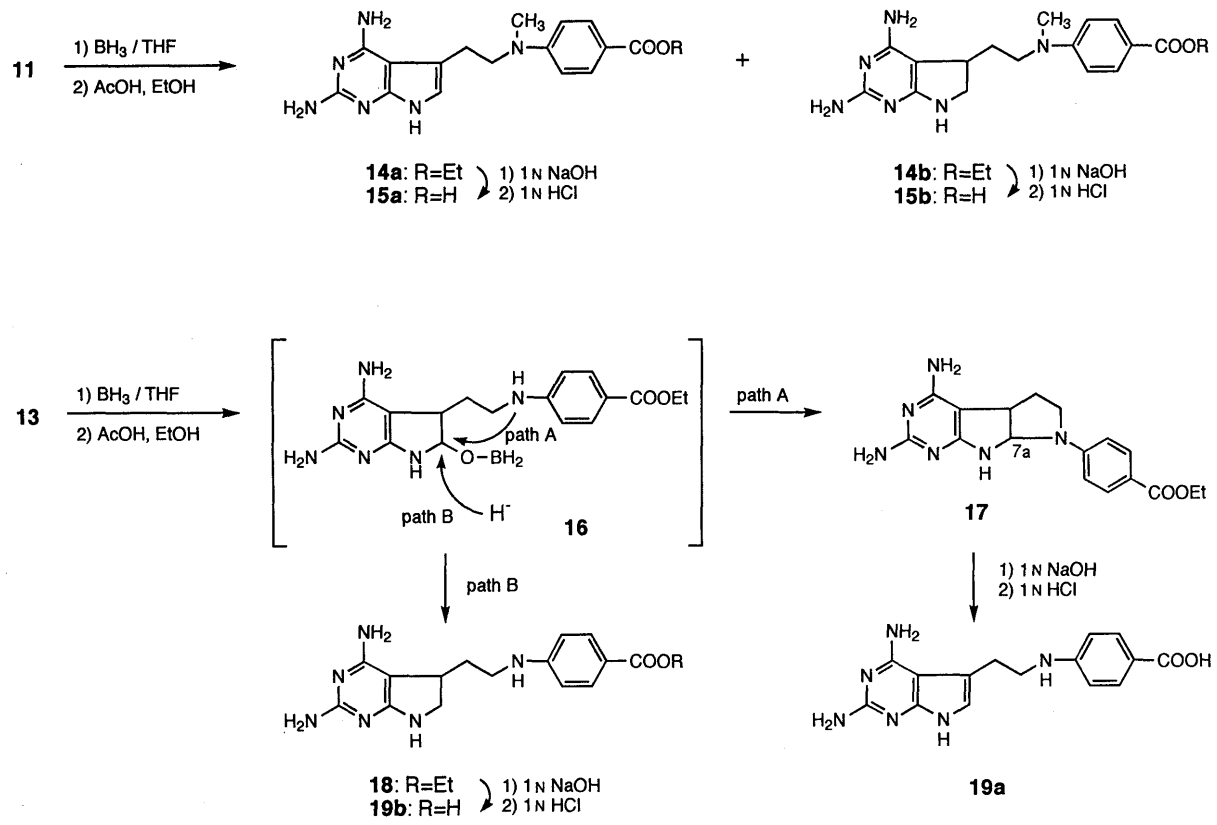

Chart 2

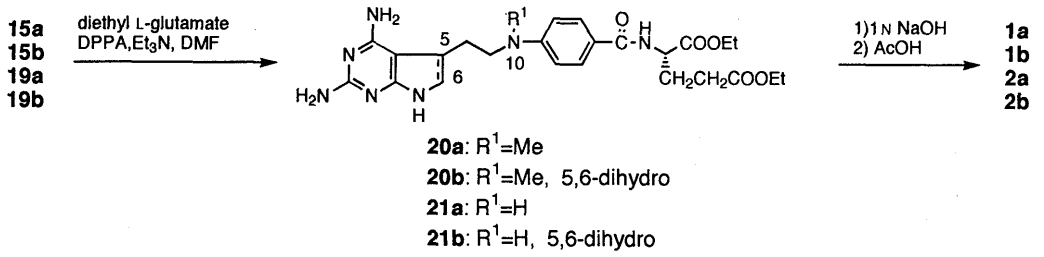

Chart 3

TABLE I. Physicochemical Data for Pyrrolo[2,3-*d*]pyrimidine Antifolates

Compound	mp (°C)	Formula[a]	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N	SI-MS (*m/z*) $(M+H)^+$
1a	178—180	$C_{21}H_{25}N_7O_5 \cdot 1.2H_2O$	52.87	5.79	20.55	52.82	5.49	20.32	456
1b	185—187	$C_{21}H_{27}N_7O_5 \cdot 1.0H_2O$	53.04	6.15	20.62	52.90	6.45	20.44	458
2a	187—190	$C_{20}H_{23}N_7O_5 \cdot 1.2H_2O$	51.88	5.53	21.17	51.93	5.58	21.20	442
2b	217—220	$C_{20}H_{25}N_7O_5 \cdot 1.0H_2O$	52.05	5.90	21.25	51.78	5.89	21.11	444

a) Each compound was dried in a vacuum over P_2O_5 at 60 °C for 5 h.

The N^{10}-methyl analogue **1b** showed an 8-fold increase of DHFR-inhibitory potency over TNP-351, and **1a** and **2a, b** exhibited potencies comparable to that of TNP-351. Thus, with regard to DHFR inhibition, the introduction of a nitrogen atom into the bridge chain resulted in a small improvement, but the potency of the compounds thus prepared did not reach the level of MTX. In the cases of **1b** and **2b**, the possibility that one of the two diastereomers might show more potent DHFR-inhibitory activity than the other remains to be examined.

However, the antifolates **1a, b** and **2a, b** markedly inhibited the proliferation of KB and A549 cells despite their modest DHFR-inhibitory potency. Because their biological activities showed a strong resemblance to those

TABLE II. Inhibition of DHFR and Tumor Cell Growth

Compound	IC_{50}		
	Inhibition of DHFR (μM)	Inhibition of tumor cell growth (ng/ml)	
		KB	A549
1a	0.21	1.3	5.0
1b	0.049	2.5	20
2a	0.99	5.0	20
2b	0.20	2.5	5.0
TNP-351	0.37	0.27	4.5
MTX	0.0055	5.0	35

of their parent compound TNP-351, as described above, they may have a similar mechanism of action to that of TNP-351; that is, i) they can be efficiently transported into tumor cells *via* the reduced folate carrier protein, ii) they are good substrates for FPGS, iii) their polyglutamates are pooled within the tumor cells, and iv) they exhibit potent inhibitory activity against the target enzyme (DHFR) and their polyglutamates also inhibit phosphoribosylaminoimidazolecarboxamide formyltransferase (AICARTF).[7-9]

In conclusion, four novel pyrrolo[2,3-*d*]pyrimidine antifolates substituted with a nitrogen at the C-10 position were synthesized by an efficient method and have been shown to possess more potent inhibitory activities than MTX against the proliferation of KB and A549 cells. This modification gave compounds which showed little improvement in DHFR-inhibitory potency. It is still necessary to achieve isolation and biological examination of 5-(*R*) and 5-(*S*) diastereomers of **1b** and **2b** in order to study the structure–activity relationships in detail. The pyrrolo[2,3-*d*]pyrimidine ring is presumed to play a decisive role in the biochemical properties of the pyrrolo-[2,3-*d*]pyrimidine antifolates rather than the bridge chain moiety. Further modifications of pyrrolo[2,3-*d*]pyrimidine antifolates are in progress in our laboratories.

Experimental

Melting points were determined on a Yanagimoto micro melting apparatus and are uncorrected. ^{1}H-NMR spectra were recorded on a Varian Gemini-200 (200 MHz) instrument with tetramethylsilane as an internal standard. IR spectra were obtained on a JASCO IR-810 IR spectrophotometer. Secondary ionization mass spectra (SI-MS) were measured with a Hitachi M-80A mass spectrometer. Elemental analyses were carried out by Takeda Analytical Research Laboratories, Ltd. The $[\alpha]_D$ values were determined in the indicated solvents on a JASCO DIP-370 instrument. Flash chromatography was performed on Merck Silica gel 60 (230—400 mesh).

Methyl 4-[*N*-(4-Ethoxycarbonylphenyl)-*N*-methylamino]butyrate (5) In an argon atmosphere, methyl 4-bromocrotonate (9.45 g, 53 mmol) was added to a suspension of ethyl 4-(methylamino)benzoate (7.89 g, 44 mmol) and K_2CO_3 (7.30 g, 53 mmol) in DMPU (22 ml). The reaction mixture was stirred for 13 h at 80 °C, then cooled to room temperature and poured into water (660 ml). The aqueous layer was extracted with ether (500 ml × 3), and the organic extracts were combined, washed with brine, dried (Na_2SO_4), and concentrated. Flash chromatography of the crude product (AcOEt–hexane, 1 : 11) gave 9.80 g (80%) of methyl 4-[*N*-(4-ethoxycarbonylphenyl)-*N*-methylamino]crotonate as a colorless oil. A solution of the above crotonate (9.80 g) was prepared in EtOH–THF (1.6 : 1) (290 ml), then AcOH (3.5 ml) and Raney Ni (W-2) (890 mg) was added, and the suspension was stirred vigorously in a hydrogen atmosphere for 30 min. The slurry was filtered through Celite, and the filtrate was concentrated to give 9.80 g (99%) of **5** as a colorless oil. IR (neat): 2950, 1740 (CO), 1700 (CO), 1600 (Ph), 1525 (Ph) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.36 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.73—2.07 (2H, m, 3-H_2), 2.34 (2H, t, J=7 Hz, 2-H_2), 3.00 (3H, s, NCH_3), 3.42 (2H, t, J=7 Hz, 4-H_2), 3.67 (3H, s, $COOCH_3$), 4.31 (2H, q, J=7 Hz, $COOCH_2C\underline{H}_3$), 6.65 (2H, d, J=9 Hz, ArH), 7.91 (2H, d, J=9 Hz, ArH).

Ethyl 4-[*N*-(*tert*-Butoxycarbonyl)-*N*-(4-ethoxycarbonylphenyl)amino]-butyrate (6) In an argon atmosphere, ethyl 4-(*tert*-butoxycarbonylamino)benzoate[15] (18.6 g, 70 mmol) was added to a suspension of NaH (60% dispersion in mineral oil, 2.02 g, 84 mmol) in DMPU (70 ml) and the mixture was stirred for 2 h at room temperature. Then NaI (12.6 g, 84 mmol) and ethyl 4-bromobutyrate (17.3 g, 84 mmol) in DMPU (15 ml) were added. The reaction mixture was stirred for a further 17 h, and poured into water (500 ml). The aqueous layer was extracted with ether (500 ml × 2), and the organic extracts were combined, washed with water and brine, dried (Na_2SO_4), and concentrated. Flash chromatography of the crude product (AcOEt–hexane, 1 : 9) gave 10.5 g (40%) of **6** as a colorless oil. IR (neat): 2980, 2930, 1740 (CO), 1710 (CO), 1600 (Ph), 1510 (Ph), 865 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.23 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.40 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.45 (9H, s, *tert*-Bu), 1.88 (2H, m, 3-H_2), 2.32 (2H, t, J=7 Hz, 2-H_2), 3.74 (2H, t, J=7 Hz, 4-H_2), 4.10 (2H, q, J=7 Hz, $COC\underline{H}_2CH_3$), 4.38 (2H, q, J=7 Hz, $COC\underline{H}_2CH_3$), 7.30 (2H, d, J=9 Hz, ArH), 8.02 (2H, d, J=9 Hz, ArH).

Methyl 4-[*N*-(4-Ethoxycarbonylphenyl)-*N*-methylamino]-2-iodobutyrate (7) In an argon atmosphere, *n*-BuLi (38.2 mmol) in hexane (25 ml) was added to a solution of diisopropylamine (3.86 g, 38.2 mmol) in THF (35 ml) at 0 °C. After 10 min of stirring, the mixture was cooled to −78 °C, and **5** (8.90 g, 32 mmol) in THF (35 ml) was added dropwise over 15 min. The resulting mixture was stirred for 30 min at −78 °C and iodine (8.08 g, 32 mmol) in THF (50 ml) was added at a temperature below −60 °C. Stirring was continued for 20 min at −78 °C, and the solution was allowed to warm to 0 °C in 30 min. After addition of 1 N aqueous $KHSO_4$ (50 ml), the resulting aqueous phase was extracted with ether (200 ml × 3). The combined extracts were washed with 1 N aqueous K_2CO_3 (50 ml × 2) and brine, dried over $MgSO_4$, and evaporated. Purification of the residue by flash chromatography (AcOEt–hexane, 1 : 19) gave 4.78 g (37%) of **7** as a white solid, mp 87—88 °C (hexane–AcOEt). IR (KBr): 2990, 2910, 1720 (CO), 1695 (CO), 1600 (Ph), 1520 (Ph), 835 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.35 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 2.13—2.43 (2H, m, 3-H_2), 3.00 (3H, s, NCH_3), 3.37—3.57 (2H, m, 4-H_2), 3.73 (3H, s, $COOCH_3$), 4.31 (2H, q, J=7 Hz, $COC\underline{H}_2CH_3$), 4.33 (1H, t, J=7 Hz, 2-H), 6.63 (2H, d, J=9 Hz, ArH), 7.88 (2H, d, J=9 Hz, ArH). *Anal.* Calcd for $C_{15}H_{20}INO_4$: C, 44.46; H, 4.97; N, 3.46. Found: C, 44.50; H, 4.93; N, 3.43.

Ethyl 4-[*N*-(*tert*-Butoxycarbonyl)-*N*-(4-ethoxycarbonylphenyl)amino]-2-iodobutyrate (8) In a similar manner to that described above for **7**, **6** (1.14 g, 3.0 mmol) was converted to **8** (0.58 g, 38%) as a pale brown oil. IR (neat): 2980, 2930, 1725 (CO), 1710 (CO), 1600 (Ph), 1510 (Ph), 860 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.25 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.40 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.45 (9H, s, *tert*-Bu), 2.13—2.37 (2H, m, 3-H_2), 3.60—3.93 (2H, m, 4-H_2), 4.17 (2H, q, J=7 Hz, $COC\underline{H}_2CH_3$), 4.31 (1H, t, J=7 Hz, 2-H), 4.38 (2H, q, J=7 Hz, $COC\underline{H}_2CH_3$), 7.28 (2H, d, J=9 Hz, ArH), 8.03 (2H, d, J=9 Hz, ArH).

Methyl 2-(Dicyanomethyl)-4-[*N*-(4-ethoxycarbonylphenyl)-*N*-methylamino]butyrate (9) A suspension of NaH (60% dispersion in mineral oil, 822 mg, 34.3 mmol) in DMSO (14 ml) was stirred at 70 °C for 1 h and cooled. To the resulting mixture was added malononitrile (2.26 g, 34.3 mmol) in DMSO (5 ml) at 0 °C. The whole was stirred for 5 min, then a solution of **7** (5.58 g, 13.7 mmol) in DMSO (50 ml) was added, and the reaction mixture was stirred for 1.5 h at room temperature. The reaction was quenched with 1 N aqueous $KHSO_4$ (100 ml), and the aqueous layer was extracted with ether (100 ml × 3). The combined ether layer was washed with water (100 ml × 3) and brine, dried ($MgSO_4$), and concentrated to a residue under reduced pressure. The residue was purified by flash chromatography (AcOEt–hexane, 1 : 8) to afford 3.68 g (78%) of **9** as a white solid, mp 94—95 °C (hexane–AcOEt). IR (KBr): 2980, 2920, 2250 (CN), 1745 (CO), 1730 (CO), 1600 (Ph), 1525 (Ph), 950 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.37 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 2.10—2.34 (2H, m, 3-H_2), 2.95—3.17 (1H, m, $CH(CN)_2$), 3.03 (3H, s, NCH_3), 3.60 (2H, t, J=7 Hz, 4-H_2), 3.85 (3H, s, $COOCH_3$), 4.17 (1H, d, J=7 Hz, 2-H), 4.33 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 6.67 (2H, d, J=9 Hz, ArH), 7.93 (2H, d, J=9 Hz, ArH). *Anal.* Calcd for

$C_{18}H_{21}N_3O_4$: C, 62.96; H, 6.16; N, 12.24. Found: C, 62.85; H, 6.19; N, 12.03.

Ethyl 4-[*N*-(*tert*-Butoxycarbonyl)-*N*-(4-ethoxycarbonylphenyl)amino]-2-(dicyanomethyl)butyrate (10) In a similar manner to that described for **9**, **8** (4.84 g, 9.57 mmol) was converted to **10** (3.47 g, 82%) as a colorless oil. IR (neat): 2980, 2930, 2250 (CN), 1740 (CO), 1710 (CO), 1600 (Ph), 1515 (Ph), 880 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.30 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.40 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.43 (9H, s, *tert*-Bu), 1.95—2.29 (2H, m, 3-H_2), 3.07 (1H, q, J=6 Hz, $CH(CN)_2$), 3.69—4.05 (2H, m, 4-H_2), 4.28 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 4.39 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 4.58 (1H, d, J=6 Hz, 2-H), 7.26 (2H, d, J=9 Hz, ArH), 8.06 (2H, d, J=9 Hz, ArH).

Ethyl 4-[*N*-[2-(2,4-Diamino-6-oxo-6,7-dihydro-5*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-*N*-methylamino]benzoate (11) Sodium metal (320 mg, 13.9 mmol) was dissolved in EtOH (30 ml) in an argon atmosphere. To the resulting EtONa solution were added **9** (3.68 g, 10.7 mmol) and guanidine hydrochloride (1.23 g, 12.9 mmol) in EtOH–THF (2 : 1, 45 ml), and the mixture was refluxed for 3 h. It was then cooled to room temperature, and poured into water (450 ml), which resulted in the formation of a precipitate. This was collected by filtration, washed with MeOH and ether, and vacuum-dried to gave 2.91 g (74%) of **11** as a white crystalline solid, mp 254—260 °C (dried in a vacuum over P_2O_5 at 60 °C for 5 h). IR (KBr): 3420 (NH_2), 3350 (CONH), 3200, 1685 (CO), 1630 (CONH), 1600 (Ph), 1580, 1520 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.27 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.90—2.23 (2H, m, 8-H_2), 2.89 (3H, s, NCH_3), 3.10—3.47 (3H, m, 5-H, 9-H_2), 4.20 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 5.87 (2H, s, NH_2), 6.08 (2H, s, NH_2), 6.63 (2H, d, J=9 Hz, ArH), 7.72 (2H, d, J=9 Hz, ArH), 10.50 (1H, s, 7-NH). *Anal.* Calcd for $C_{18}H_{22}N_6O_3 \cdot 0.3H_2O$: C, 57.53; H, 6.06; N, 22.36. Found: C, 57.77; H, 6.12; N, 22.17.

Ethyl 4-[*N*-[2-(2,4-Diamino-6-oxo-6,7-dihydro-5*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-*N*-(*tert*-butoxycarbonyl)amino]benzoate (12) In a similar manner to that described for **11**, **10** (3.45 g, 9.34 mmol) was converted to **12** (3.00 g, 85%) as a white solid, mp 230—231 °C (dried in a vacuum over P_2O_5 at 60 °C for 5 h). IR (KBr): 3450 (NH_2), 3360 (CONH), 3230, 2980, 1715m (CO), 1690 (CO), 1635 (CONH), 1605 (Ph) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.32 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.39 (9H, s, *tert*-Bu), 1.90—2.30 (2H, m, 8-H_2), 3.30 (1H, t, J=6 Hz, 5-H), 3.39—3.70 (2H, m, 9-H_2), 4.31 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 5.68 (2H, s, NH_2), 6.03 (2H, s, NH_2), 7.39 (2H, d, J=9 Hz, ArH), 7.90 (2H, d, J=9 Hz, ArH), 10.50 (1H, s, 7-NH). *Anal.* Calcd for $C_{22}H_{28}N_6O_5 \cdot 0.5H_2O$: C, 56.76; H, 6.28; N, 18.05. Found: C, 57.06; H, 6.18; N, 18.09.

Ethyl 4-[*N*-[2-(2,4-Diamino-6-oxo-6,7-dihydro-5*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]amino]benzoate (13) Compound **12** (2.93 g, 6.40 mmol) was dissolved with stirring in TFA (30 ml), and after 1 h the TFA was evaporated. The residue was taken up in water (60 ml) and the solution was brought to pH 7.5 with 0.1 N aqueous NaOH. The precipitate was filtered off, washed with water, EtOH, and ether, and dried in a vacuum over P_2O_5 at 60 °C for 5 h to give 2.00 g (86%) of **13** as a white solid, mp 237—240 °C. IR (KBr): 3470 (NH_2), 3380 (CONH), 3170, 2980, 1690 (CO), 1650 (CONH), 1600 (Ph), 1580, 1530 cm^{-1}. ^{1}H-NMR (DMSO-d_6 + D_2O) δ: 1.27 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 2.02—2.18 (2H, m, 8-H_2), 2.92—3.13 (2H, m, 9-H_2), 3.40 (1H, t, J=6 Hz, 5-H), 4.21 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 6.54 (2H, d, J=9 Hz, ArH), 7.68 (2H, d, J=9 Hz, ArH). *Anal.* Calcd for $C_{17}H_{22}N_6O_3 \cdot 0.8H_2O$: C, 55.07; H, 5.87; N, 22.66. Found: C, 55.02; H, 6.02; N, 22.41.

Ethyl 4-[*N*-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-*N*-methylamino]benzoate (14a) and Ethyl 4-[*N*-[2-(2,4-Diamino-6,7-dihydro-5*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-*N*-methylamino]benzoate (14b) A suspension of **11** (2.75 g, 7.42 mmol) in THF (40 ml) was treated with 1.0 M BH_3–THF in THF (59.4 ml) under argon at 0 °C, and the resulting solution was stirred for 6 h. The reaction was quenched by addition of AcOH–EtOH (1 : 2, 90 ml), and the mixture was left to stand for 18 h at room temperature, then evaporated *in vacuo*. The residue was purified by flash chromatography (CH_2Cl_2–8% NH_3 in EtOH, eluent gradient 33 : 1 to 25 : 1) to give initially 853 mg (32%) of **14a** and then 1.32 g (50%) of **14b**. Each product was a white solid. **14a**: mp 208—210 °C (EtOH, dried in a vacuum over P_2O_5 at 60 °C for 5 h). IR (KBr): 3400 (NH_2), 3150, 1695 (CO), 1640, 1605 (Ph), 1570, 1530 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.28 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 2.90 (2H, t, J=7 Hz, 8-H_2), 2.93 (3H, s, NCH_3), 3.61 (2H, t, J=7 Hz, 9-H_2), 4.22 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 5.40 (2H, s, NH_2), 6.02 (2H, s, NH_2), 6.45 (1H, s, 6-H), 6.70 (2H, d, J=9 Hz, ArH), 7.74 (2H, d, J=9 Hz, ArH), 10.46 (1H, s, 7-NH). *Anal.* Calcd for $C_{18}H_{24}N_6O_2 \cdot 0.3EtOH$: C, 60.67; H, 6.51; N, 22.82. Fouund: C, 60.68; H, 6.42; N, 22.70. **14b**: mp 208—212 °C (EtOH, dried in a vacuum over P_2O_5 at 60 °C for 5 h). IR (KBr): 3350 (NH_2), 3150, 2980, 1675 (CO), 1620, 1590 (Ph), 1520 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.28 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.45—1.68 (1H, m, 8-H), 1.71—1.92 (1H, m, 8-H), 2.95 (3H, s, NCH_3), 3.05—3.22 (2H, m, 5-H, 6-H), 3.32—3.54 (3H, m, 6-H, 9-H_2), 4.22 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 5.38 (2H, s, NH_2), 5.56 (2H, s, NH_2), 6.00 (1H, s, 7-NH), 6.72 (2H, d, J=9 Hz, ArH), 7.74 (2H, d, J=9 Hz, ArH). *Anal.* Calcd for $C_{18}H_{24}N_6O_2 \cdot 0.2EtOH$: C, 60.44; H, 6.95; N, 22.98. Found: C, 60.33; H, 6.86; N, 23.03.

Ethyl 4-(2,4-Diamino-4b,5,6,7,7a,8-hexahydropyrrolo[3′,2′ : 4,5]pyrrolo-[2,3-*d*]pyrimidin-7-yl]benzoate (17) and Ethyl 4-[*N*-[2-(2,4-Diamino-6,7-dihydro-5*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)-ethyl]amino]benzoate (18) By a similar procedure to that described above for **14a**, **b**, **13** (1.00 g, 2.80 mmol) was converted to 563 mg (59%) of **17** as a white solid and 230 mg (24%) of **18** as an amorphous powder. **17**: mp 163—166 °C (EtOH, dried in a vacuum over P_2O_5 at 60 °C for 5 h). IR (KBr) 3400 (NH_2), 3200, 1695 (CO), 1630, 1610 (Ph), 1520 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.30 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.89—2.06 (1H, m, 5-H), 2.14—2.35 (1H, m, 5-H), 3.08—3.27 (1H, m, 6-H), 3.42—3.57 (1H, m, 6-H), 3.76—3.89 (1H, m, 6a-H), 4.24 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 5.45 (2H, s, NH_2), 5.62 (1H, d, J=8 Hz, 7a-H), 5.69 (2H, s, NH_2), 6.79 (2H, d, J=9 Hz, ArH), 7.14 (1H, s, 8-NH), 7.75 (2H, d, J=9 Hz, ArH). *Anal.* Calcd for $C_{17}H_{20}N_6O_2 \cdot 0.3EtOH$: C, 59.68; H, 6.20; N, 23.73. Found: C, 59.44; H, 6.18; N, 23.79. **18**: mp 233—235 °C (EtOH). IR (KBr) 3390 (NH_2), 3200, 2940, 1680 (CO), 1600 (Ph), 1585, 1535 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.27 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.47—1.68 (1H, m, 8-H), 1.77—1.97 (1H, m, 8-H), 3.00—3.23 (4H, m, 5-H, 6-H, 9-H_2), 3.44 (1H, t, J=10 Hz, 6-H), 4.20 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 5.36 (2H, s, NH_2), 5.50 (2H, s, NH_2), 5.97 (1H, s, 7-NH), 6.48 (1H, t, J=5 Hz, 10-NH), 6.58 (2H, d, J=9 Hz, ArH), 7.69 (2H, d, J=9 Hz, ArH).

Diethyl 4-[*N*-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-*N*-methylamino]benzoyl-L-glutamate (20a) The pyrrolopyrimidine derivative **14a** (621 mg, 1.75 mmol) was stirred for 12 h at 60 °C in a mixture of 1 N aqueous NaOH (8.75 ml) and MeOH–H_2O (1 : 1, 30 ml). After addition of 1 N aqueous HCl (8.75 ml), evaporation of the solvent and vacuum-drying gave 4-[*N*-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-*N*-methylamino]benzoic acid (**15a**) as a white amorphous solid, which was used in the following reaction without further purification. To a suspension of the above **15a** and diethyl L-glutamate hydrochloride (630 mg, 2.63 mmol) in *N*,*N*-dimethylformamide (DMF) (10 ml) was added a solution of DPPA (578 mg, 2.10 mmol) in DMF (5 ml) at 0 °C, and the mixture was stirred for 15 min. After addition of triethylamine (582 mg, 5.25 mmol) in DMF (5 ml), stirring was continued for 30 min at 0 °C and then for 72 h at room temperature. The slurry was filtered and the filtrate was concentrated to a residue, which was then purified by flash chromatography (CH_2Cl_2–8% NH_3 in EtOH, eluent gradient 33 : 1 to 25 : 1) to give 485 mg (54%) of **20a** as an amorphous powder, mp 92—96 °C (EtOH), $[\alpha]_D^{20}$ −11.4° (c=0.10, MeOH). IR (KBr): 3380 (NH_2), 2930, 1735 (CO), 1635 (CONH), 1600 (Ph), 1575, 1510 cm^{-1}. ^{1}H-NMR ($CDCl_3$ + CD_3OD) δ: 1.24 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.31 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 2.05—2.41 (2H, m, Glu-β-H_2), 2.43—2.58 (2H, m, 8-H_2), 2.92 (3H, s, NCH_3), 2.95 (2H, t, J=7 Hz, Glu-γ-H_2), 3.71 (2H, t, J=7 Hz, 9-H_2), 4.13 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 4.24 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 4.71—4.80 (1H, m, Glu-α-H), 6.53 (1H, s, 6-H), 6.70 (2H, d, J=9 Hz, ArH), 7.73 (2H, d, J=9 Hz, ArH).

Diethyl 4-[*N*-[2-(2,4-Diamino-6,7-dihydro-5*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-*N*-methylamino]benzoyl-L-glutamate (20b) According to the procedure described above for **20a**, **14b** (891 mg, 2.50 mmol) afforded **20b** (737 mg, 57%) as an amorphous powder *via* 4-[*N*-[2-(2,4-diamino-6,7-dihydro-5*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-*N*-methylamino]benzoic acid (**15b**). **20b** (a mixture of two diastereomers): mp 83—86 °C (EtOH). IR (KBr): 3370 (NH_2), 3200, 2980, 2930, 1730 (CO), 1630 (CONH), 1600 (Ph), 1510 cm^{-1}. ^{1}H-NMR ($CDCl_3$ + CD_3OD) δ: 1.23 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.31 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.75—1.97 (2H, m, Glu-β-H_2), 2.06—2.39 (2H, m, 8-H_2), 2.43—2.54 (2H, m, Glu-γ-H_2), 2.99 (3H, s, NCH_3), 3.03—3.15 (2H, m, 5-H, 6-H), 3.20—3.48 (2H, m, 9-H_2), 3.64—3.80 (1H, m, 6-H), 4.12 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 4.23 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 4.71—4.78 (1H, m, Glu-α-H), 6.67 (2H, d, J=9 Hz, ArH), 7.71 (2H, d, J=9 Hz, ArH).

Diethyl 4-[*N*-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-amino]benzoyl-L-glutamate (21a) The tricyclic compound **17** (475 mg, 1.40 mmol) was stirred for 12 h at 60 °C in a mixture of 1 N aqueous NaOH (1.76 ml) and MeOH–H_2O (1 : 1, 16 ml). After addition of 1 N aqueous HCl (1.76 ml), evaporation of the solvent and vacuum-drying gave 4-[*N*-[2-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]amino]benzoic acid (**19a**) as an amorphous powder, which was used in the following reaction without further purification. Compound **19a** was reacted with diethyl L-glutamate in a manner similar to that described above for **20a** to give 522 mg (75%) of **21a** as an amorphous powder, mp 91—93 °C (EtOH), $[\alpha]_D^{20}$ −11.4° (c=0.21, MeOH). IR (KBr): 3400 (NH_2), 1740 (CO), 1605 (Ph), 1575, 1545, 1515 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.17 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.18 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.87—2.20 (2H, m, Glu-β-H_2), 2.41 (2H, t, J=8 Hz, 8-H_2), 2.92 (2H, t, J=8 Hz, Glu-γ-H_2), 3.29 (2H, t, J=8 Hz, 9-H_2), 4.05 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 4.09 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 4.32—4.46 (1H, m, Glu-α-H), 5.36 (2H, s, NH_2), 5.97 (2H, s, NH_2), 6.12—6.22 (1H, m, 10-NH), 6.51 (1H, d, J=1 Hz, 6-H), 6.58 (2H, d, J=9 Hz, ArH), 7.67 (2H, d, J=9 Hz, ArH), 8.23 (1H, d, J=7 Hz, CONH), 10.45 (1H, d, J=1 Hz, 7-NH).

Diethyl 4-[*N*-[2-(2,4-Diamino-6,7-dihydro-5*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]amino]benzoyl-L-glutamate (21b) According to the procedure described above for **20a**, **18** (100 mg, 0.29 mmol) afforded **21b** (61 mg, 42%) as an amorphous powder *via* 4-[*N*-[2-(2,4-diamino-6,7-dihydro-5*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]amino]benzoic acid (**19b**). **21b** (a mixture of two diastereomers): mp 88—90 °C (EtOH). IR (KBr): 3380 (NH_2), 2980, 1740 (CO), 1635 (CONH), 1605 (Ph), 1510 cm^{-1}. ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 1.23 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.30 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$), 1.71—2.05 (2H, m, Glu-β-H_2), 2.06—2.34 (2H, m, 8-H_2), 2.35—2.55 (2H, m, Glu-γ-H_2), 3.06—3.46 (4H, m, 5-H, 6-H, 9-H_2), 3.72 (1H, t, J=9 Hz, 6-H), 4.11 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 4.23 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 4.72—4.83 (1H, m, Glu-α-H), 6.59 and 6.60 (2H, d, J=9 Hz, ArH), 7.66 and 7.67 (2H, d, J=9 Hz, ArH).

4-[*N*-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-*N*-methylamino]benzoyl-L-glutamic Acid (1a) A solution of **20a** (389 mg, 0.76 mmol) in THF–water (3 : 2, 15 ml) was treated with 1 N aqueous NaOH (2.28 ml) at room temperature, and the mixture was stirred for 3 h. It was then concentrated to 5 ml *in vacuo*, and AcOH (0.5 ml) was added dropwise to the residue. The white crystalline precipitate was collected by filtration, washed successively with water, MeOH, and ether, and dried over P_2O_5 to give 311 mg (90%) of **1a**, $[\alpha]_D^{20}$ +13.6° (c=0.090, 0.1 N NaOH). IR (KBr): 3330 (NH_2), 3200, 2930, 1690 (CO), 1670 (CO), 1630 (CONH), 1600 (Ph), 1540, 1500 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.87—2.17 (2H, m, Glu-β-H_2), 2.34 (2H, t, J=7 Hz, 8-H_2), 2.89 (2H, t, J=7 Hz, Glu-γ-H_2), 2.92 (3H, m, NCH_3), 3.60 (2H, t, J=7 Hz, 9-H_2), 4.31—4.44 (1H, m, Glu-α-H), 5.51 (2H, s, NH_2), 6.13 (2H, s, NH_2), 6.47 (1H, s, 6-H), 6.68 (2H, d, J=9 Hz, ArH), 7.74 (2H, d, J=9 Hz, ArH), 8.71 (1H, d, J=7 Hz, CONH), 10.53 (1H, s, 7-NH).

4-[*N*-[2-(2,4-Diamino-6,7-dihydro-5*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)-ethyl]-*N*-methylamino]benzoyl-L-glutamic Acid (1b) This compound was obtained as a white microcrystalline powder (373 mg 91%) by alkaline hydrolysis of **20b** (463 mg, 0.90 mmol), as described above for the preparation of **1a**. **1b** (a mixture of two diastereomers): IR (KBr): 3350 (NH_2), 3200, 2930, 1690 (CO), 1675 (CO), 1630 (CONH), 1600 (Ph), 1510 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.42—1.66 (1H, m, 8-H), 1.75—2.09 (3H, m, 8-H, Glu-β-H_2), 2.29 (2H, t, J=7 Hz, Glu-γ-H_2), 2.93 (3H, s, NCH_3), 3.13—3.46 (4H, m, 5-H, 6-H, 9-H_2), 3.52-3.65 (1H, m, 6-H), 4.24—4.37 (1H, m, Glu-α-H), 6.19—6.45 (5H, m, $2NH_2$, 7-NH), 6.70 (2H, d, J=9 Hz, ArH), 6.79 (1H, m, 10-NH), 7.69 (2H, d, J=9 Hz, ArH), 8.04 and 8.08 (1H, d, J=7 Hz, CONH).

4-[*N*-[2-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]amino]-benzoyl-L-glutamic Acid (2a) This compound was obtained as a white microcrystalline powder (220 mg, 87%) by alkaline hydrolysis of **21a** (286 mg, 0.57 mmol), as described above for the preparation of **1a**. **2a**: $[\alpha]_D^{20}$ +16.9° (c=0.38, 0.1 N NaOH). IR (KBr): 3360 (NH_2), 3210, 1690 (CO), 1640 (CONH), 1605 (Ph), 1510 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.86—2.14 (2H, m, Glu-β-H_2), 2.34 (2H, t, J=7 Hz, 8-H_2), 2.93 (2H, t, J=7 Hz, Glu-γ-H_2), 3.28 (2H, t, J=7 Hz, 9-H_2), 4.30—4.38 (1H, m, Glu-α-H), 5.51 (2H, m, NH_2), 6.12 (2H, m, NH_2), 6.55 (1H, s, 6-H), 6.58 (2H, d, J=9 Hz, ArH), 7.67 (2H, d, J=9 Hz, ArH), 8.10 (1H, m, CONH), 10.52 (1H, m, 7-NH).

4-[*N*-[2-(2,4-Diamino-6,7-dihydro-5*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)-ethyl]amino]benzoyl-L-glutamic Acid (2b) This compound was obtained as a white microcrystalline powder (23 mg, 45%) by alkaline hydrolysis of **21b** (57 mg, 0.11 mmol), as described above for the preparation of **1a**. **2b** (a mixture of two diastereomers): IR (KBr): 3360 (NH_2), 3150, 1675 (CO), 1605 (Ph), 1575, 1510 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.50—1.72 (1H, m, 8-H), 1.78—2.12 (3H, m, 8-H, Glu-β-H_2), 2.30 (2H, t, J=7 Hz, Glu-γ-H_2), 3.00—3.15 (2H, m, 5-H, 6-H), 3.17—3.33 (2H, m, 9-H_2), 3.48-3.61 (1H, m, 6-H), 4.23—4.34 (1H, m, Glu-α-H), 6.15 (3H, m, NH_2, 7-NH), 6.26 (2H, m, NH_2), 6.58 (2H, d, J=9 Hz, ArH), 6.70 (1H, m, 10-NH), 7.65 (2H, d, J=9 Hz, ArH), 7.81 (1H, m, CONH).

Growth Inhibition Assay Human epidermoid carcinoma KB cells (1×10^4 cells/ml) and human lung carcinoma A549 cells (1×10^4 cells/ml) were inoculated into wells of a 96-microwell plate and cultured in MEM medium containing 10% fetal calf serum at 37 °C under 5% CO_2 for 24 h. To this culture was added a solution of each pyrrolopyrimidine antifolate in 10% MEM at final concentrations of 0.15—80 ng/ml. Culture was continued at 37 °C under CO_2 for 72 h, and the number of cells was estimated by the MTT method[16] to determine the concentration required for 50% inhibition of growth (IC_{50}).

DHFR Inhibition Assay DHFR activity was measured by a modification of the optical photometric method reported by Bertino *et al.*[17] The reaction was carried out in 300 μl of reaction buffer containing 0.1 M Tris–HCl (pH 7.5), 150 mM KCl, 15 mM 2-mercaptoethanol and 125 mM NADPH, 0.25 μg protein/ml (1.9 μU/ml) bovine liver DHFR and various concentrations of dihydrofolic acid as indicated, at 30 °C in a flat-bottomed 96-well plate (Nunc-Immunoplate Maxisorp) pretreated with 1 mg/ml bovine serum albumin. The reaction was started by addition of 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 with a Titertek Multiskan MCC/340 (Labsystems, Finland) controlled by a personal computer for 2 min at intervals of 2 s.

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