Antirheumatic Agents. III. Novel Methotrexate Derivatives Bearing an Indoline Ring and a Modified Ornithine or Glutamic Acid[1)]

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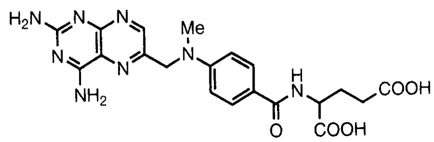
The synthesis, biological profile and structure–activity relationship of various methotrexate (MTX) derivatives bearing an indoline ring are described. In particular, N^{δ}-(3-carboxyphenyl)-N^{α}-[1-[(2,4-diaminopteridin-6-yl)-methyl]indoline-5-ylcarbonyl]-L-glutamine (3d), compared to MTX, exhibited an enhanced anti-proliferative effect on human peripheral blood mononuclear cells obtained from healthy volunteers.

Key words methotrexate; MX-9; indoline; anti-proliferation

We previously found[1)] that MX-9 (**2**), a derivative of methotrexate (MTX, **1**) bearing an indoline ring and phthaloylornithine in place of aminobenzoic acid and glutamic acid (Chart 1), respectively, potently inhibited the proliferation of human synovial cells (hSC) and peripheral blood mononuclear cells (hPBMC) from patients with rheumatoid arthritis (RA) and from healthy volunteers, respectively. In comparison with MTX, the larger amino acid moiety in MX-9 was suggested to intensify the inhibition of cell proliferation through enhanced interaction of the phthaloylornithine moiety with dihydrofolate reductase (DHFR), the target protein,[2)] presumably *via* ionic, hydrogen-bonding and/or hydrophobic interactions. Therefore, it was of considerable interest to elucidate the structure–activity relationship (SAR) for MX-9 and its derivatives to aid in the drug design of anti-folate agents.[1,2)] We therefore synthesized MX-9 derivatives, having structurally relevant amino acid moieties such as N^{δ}-acylated ornithine and N^{γ}-amidated glutamic acid (Chart 2).


methotrexate (MTX) **1**

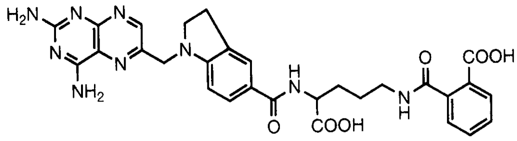

MX-9 **2**

Chart 1

In this paper, we report the synthesis, biological profile and SAR of novel MTX derivatives bearing an indoline ring and ornithine or glutamine as an amino acid moiety (Chart 2).

Chemistry

The synthetic schemes for the intermediates **8a**—**e** and the final compounds **3a**—**e** are presented in Charts 3 and 4, respectively. As shown in Chart 3, **8a**, **b** and **8c**—**e** were prepared from N^{α}-benzyloxycarbonylornithine (**4**) and

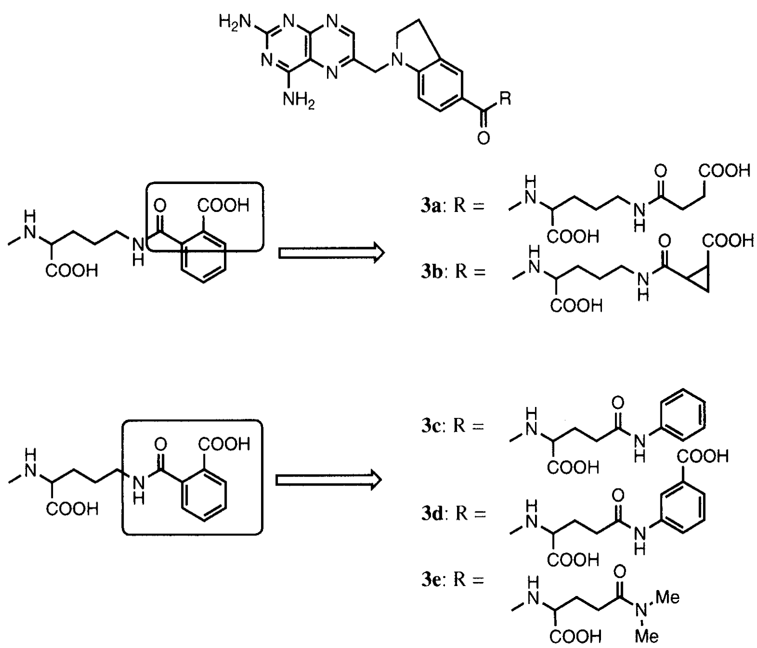


Chart 2

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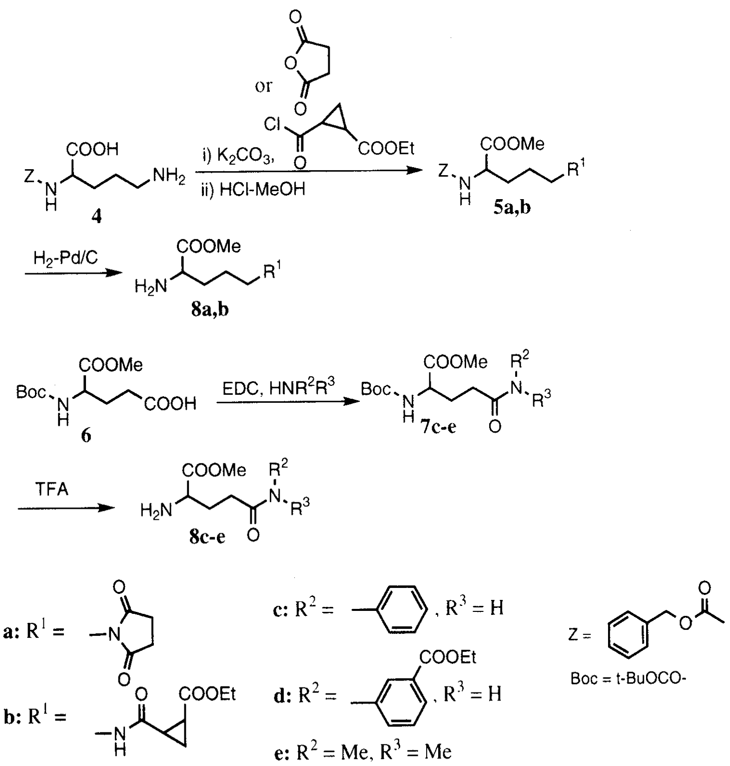

Chart 3

N-*tert*-butoxycarbonylglutamic acid α-methyl ester (**6**), respectively. Acylations of **4** were achieved by using the Schotten–Baumann procedure to give the amides **5a, b**. The carbobenzoxy groups of **5a, b** were effectively removed by means of palladium catalytic hydrogenation to yield intermediates **8a, b**. Amidation of **6** with amines was performed by treatment with 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) to give the amides **7c—e**. The *tert*-butoxy groups of **7c—e** were then removed with trifluoroacetic acid (TFA) to yield intermediates **8c—e**. As shown in Chart 4, the final compounds **3a—e** were synthesized *via* standard synthetic methods.[3] Initially, 1-benzyloxycarbonylindoline-5-carboxylic acid (**9**) was converted to the corresponding acid chloride by treatment with thionyl chloride in the presence of a catalytic amount of N,N-dimethylformamide (DMF). Subsequently, couplings with **8a—e** were performed by the Schotten–Baumann procedure to give the amides **10a—e**. The carbobenzoxy groups of **10a—e** were then removed by palladium-catalyzed hydrogenation to give deprotected amines, which were immediately alkylated with 6-bromomethyl-2,4-diaminopteridine[4] (**11**) to yield **12a—e**. These products were next hydrolyzed with 1 N NaOH to produce the final compounds **3a—e**.

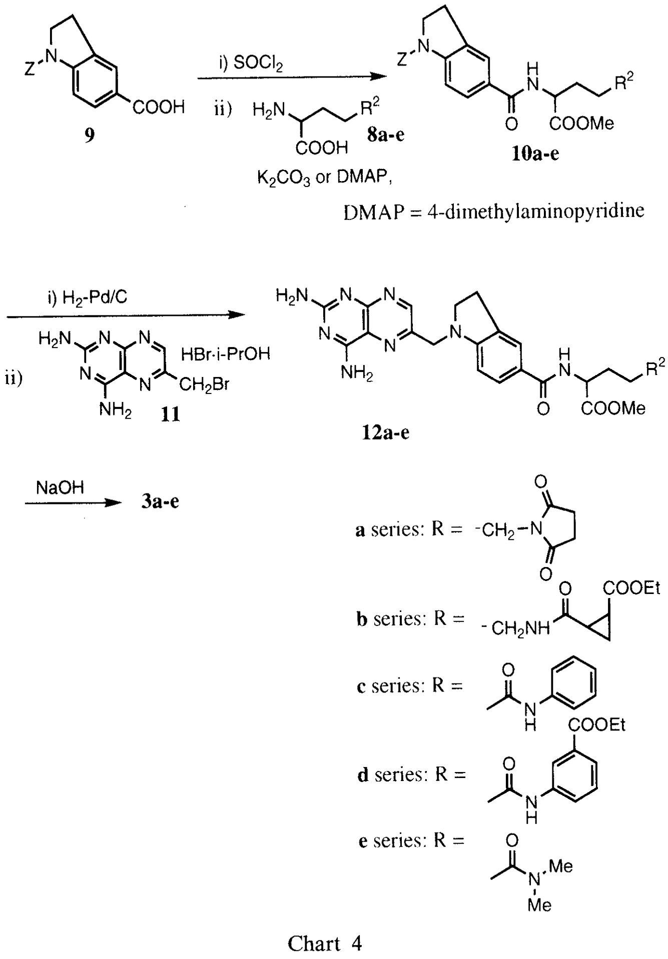

Chart 4

Results and Discussion

As shown in the table, the novel MTX derivatives were evaluated for anti-proliferative activity against hPBMC and hSC *in vitro*. In the hPBMC assay, all the tested compounds exhibited anti-proliferative activities. In particular, the glutamate derivative **3d** was 2 times more potent than MTX with an IC_{50} value of 12 nM. The anti-proliferative effects of the glutamate derivative **3c** and ornithine derivatives **3a, b** were comparable to that of MTX with IC_{50} values of 33, 59 and 42 nM, respectively. The glutamate derivative **3e**, however, was 6 times less potent than MTX with an IC_{50} value of 140 nM. These

Table 1. Anti-proliferative Activities of Novel MTX Derivatives[a)]

Compound No.	IC_{50} values (nM)	
	Human SC	Human PBMC
3a	250	59
3b	300	42
3c	6300	33
3d	540	12
3e	2200	140
MX-9 (**2**)	22	2.1
MTX	61	24

a) Anti-proliferative activities of novel MTX derivatives were determined as described in ref. 1. The results are the mean values of triplicate assays.

results suggest that the binding pocket in the target protein (presumably DHFR) is wider than had been expected from the model of DHFR binding with MTX developed by Oefner *et al.*[5)] Actually, derivatives **3a**—**d**, having greater volume in their amino acid moiety in comparison with MTX, exhibited considerable anti-proliferative effects. In the hSC assay, by contrast, all the tested compounds were effective, but were considerably less potent than MTX and MX-9. Interestingly, the anti-proliferative effects of **3a**, **3b** and **3d**, having two COOH groups, were obviously enhanced compared to those of **3c** and **3e**, each having one COOH group. This tendency was not observed in the hPBMC assay, suggesting that two COOH groups at the amino acid moiety in the MTX derivatives play a critical role in penetration of the hSC membrane.

In conclusion, we found that greater volume at the amino acid moiety of MTX derivatives contributed to the potent anti-proliferative effects, and that two COOH groups at the amino acid moiety were required for permeation of the compounds through the hSC membrane.

Experimental

NMR spectra were recorded on a JEOL JMN-FX200 NMR spectrometer with Me_4Si as the reference, infrared spectra were run on a Hitachi 270-3 spectrometer, and EI mass spectra were recorded on a Shimadzu GCMS-QP1000. FAB and high-resolution (HR)-FAB mass spectra were recorded on a VG Analytical VG11-250. TLC was routinely performed on Merck Kieselgel 60 F_{254}.

Methyl N^{α}-Benzyloxycarbonyl-N^{δ}-succinyl-L-ornithinate (5a) A mixture of N^{α}-benzyloxycarbonylornithine (5.0 g), succinic anhydride (3.8 g) and K_2CO_3 (4.0 g) in CH_2Cl_2–H_2O (1 : 1, 200 ml) was vigorously stirred overnight at room temperature, concentrated and dried *in vacuo*. To a suspension of the obtained residue in DMF (200 ml) were added MeI (10 ml) and K_2CO_3 (10 g) and the mixture was stirred for 10 h at room temperature, poured into water, and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4 and concentrated. The residue was chromatographed on silica gel with 1% MeOH in $CHCl_3$ to give **5a** (5.0 g, 73%) as a white powder. ^1H-NMR ($CDCl_3$) δ: 1.5—1.8 (4H, m), 2.63 (4H, s), 3.57 (2H, m), 3.69 (3H, s), 5.06 (2H, s), 5.56 (1H, d, J=7.3 Hz), 4.1—4.5 (1H, m), 7.2—7.4 (5H, m). HR-MS *m*/*z*: Calcd for $C_{18}H_{22}N_2O_6$: M, 362.1478. Found: 362.1483 (M^+).

Methyl N^{α}-Benzyloxycarbonyl-N^{δ}-[2-(ethoxycarbonyl)cyclopropylcarbonyl]-L-ornithinate (5b) A mixture of 2-ethoxycarbonylcyclopropane-1-carboxylic acid (3.2 g) and DMF (50 ml) in thionyl chloride (10 ml) was refluxed for 1 h and concentrated. To a solution of the above residue in CH_2Cl_2 (80 ml) were added a solution of N^{α}-benzyloxycarbonylornithine (5.0 g) and K_2CO_3 (5.2 g) in water (80 ml), and the reaction mixture was vigorously stirred for 8 h. It was then concentrated, diluted to 80 ml with water, and adjusted to pH 2 with 1 N HCl, and the resulting solids were collected by filtration, dried *in vacuo* and dissolved in dry MeOH (100 ml). To this solution was added thionyl chloride (8.0 ml) at −10 °C, and the reaction mixture was refluxed for 2 h, then concentrated. The residue was poured into 5% $NaHCO_3$ aqueous solution and extracted with $CHCl_3$, dried over Na_2SO_4, filtered, concentrated and chromatographed on silica gel with 1% MeOH in $CHCl_3$ to give **5b** (6.6 g, 84%) as a white powder. ^1H-NMR ($CDCl_3$) δ: 1.25 (3H, m), 1.3—2.2 (8H, m), 3.23 (2H, m), 3.70 (3H, s), 4.10 (2H, m), 4.32 (1H, m), 5.09 (2H, s), 5.76 (1H, m), 6.68 (1H, m), 7.30 (5H, m). IR (neat) cm^{-1}: 3400—3300, 2950, 1720, 1660, 1540. HR-MS *m*/*z*: Calcd for $C_{21}H_{28}N_2O_7$: M, 420.1896. Found: 420.1886 (M^+).

Methyl N^{α}-*tert*-Butoxycarbonyl-N^{δ}-phenyl-L-glutaminate (7c) To the solution of α-methyl *N*-*tert*-butoxycarbonyl-L-glutamate (**6**, 0.49 g) in CH_2Cl_2 (10 ml) was added aniline (0.21 ml) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 0.54 g). The mixture was stirred overnight at room temperature, diluted with $CHCl_3$ and washed with 5% $NaHCO_3$ aqueous solution. The organic layer was dried over Na_2SO_4, filtered and concentrated. The residue was chromatographed on silica gel with 3% MeOH in $CHCl_3$ to give **7c** (0.43 g, 68%) as a colorless oil. ^1H-NMR ($CDCl_3$) δ: 1.44 (9H, s), 1.8—2.1 (1H, m), 2.1—2.4 (1H, m), 2.4—2.5 (2H, m), 3.71 (3H, s), 4.3—4.4 (1H, m), 5.50 (1H, d, J=7.9 Hz), 7.08 (1H, d, J=7.8 Hz), 7.29 (2H, t, J=7.8 Hz), 7.57 (2H, d, J=7.8 Hz), 8.68 (1H, s). IR (neat) cm^{-1}: 3150, 3000, 1720, 1680, 1610. HR-MS *m*/*z*: Calcd for $C_{17}H_{24}N_2O_5$: M, 336.1685. Found: 336.1681 (M^+).

Methyl N^{α}-*tert*-Butoxycarbonyl-N^{δ}-(3-ethoxycarbonylphenyl)-L-glutaminate (7d) Using the same procedure as described for the preparation of **7c**, **7d** was prepared from **6** and ethyl 4-aminobenzoate. The yield of **7d** was 69%. Colorless oil. ^1H-NMR ($CDCl_3$) δ: 1.38 (3H, t, J=7.1 Hz), 1.47 (9H, s), 1.8—2.1 (1H, m), 2.2—2.4 (1H, m), 2.4—2.5 (2H, m), 3.74 (3H, s), 4.37 (3H, m), 5.37 (1H, d, J=7.3 Hz), 7.40 (1H, t, J=7.8 Hz), 7.78 (1H, d, J=7.8 Hz), 7.96 (1H, d, J=9.3 Hz), 8.13 (1H, s), 8.83 (1H, s). IR (neat) cm^{-1}: 1720, 1610. HR-MS *m*/*z*: Calcd for $C_{20}H_{28}N_2O_7$: M, 408.1896. Found: 408.1892 (M^+).

Methyl N^{α}-*tert*-Butoxycarbonyl-N^{δ}-dimethyl-L-glutaminate (7e) Using the same procedure as described for the preparation of **7c**, **7e** was prepared from **6** and dimethylamine hydrochloride. The yield of **7e** was 96%. Colorless oil. ^1H-NMR ($CDCl_3$) δ: 1.44 (9H, s), 1.9—2.1 (1H, m), 2.1—2.3 (1H, m), 2.3—2.5 (2H, m), 2.95 (3H, s), 2.99 (3H, s), 2.73 (3H, s), 4.2—4.4 (1H, m), 5.3—5.5 (1H, m). IR (neat) cm^{-1}: 2990, 1730, 1720, 1710, 1620. HR-MS *m*/*z*: Calcd for $C_{13}H_{24}N_2O_5$: M, 288.1685. Found: 288.1655 (M^+).

Methyl N^{δ}-Succinyl-L-ornithinate (8a) A mixture of **5a** (5.0 g) and 5% Pd on carbon (300 mg) in MeOH (100 ml) was stirred for 10 h under a hydrogen atmosphere, filtered and concentrated. The residue was chromatographed on silica gel with 5% MeOH in $CHCl_3$ to give **8a** (1.75 g, 56%) as a colorless powder. ^1H-NMR ($CDCl_3$) δ: 1.5—2.0 (4H, m), 2.71 (4H, s), 3.3—3.7 (3H, m), 3.72 (3H, s). IR (neat) cm^{-1}: 3600—3300, 2950, 1730, 1690, 1440, 1410, 1340, 1210. HR-MS *m*/*z*: Calcd for $C_{10}H_{17}N_2O_4$: MH, 229.1188. Found: 229.1196 (MH^+).

Methyl N^{δ}-[2-(Ethoxycarbonyl)cyclopropylcarbonyl]-L-ornithinate (8b) Using the same procedure as described for the preparation of **8a**, **8b** was prepared from **5b**. The yield of **8b** was 76%. Colorless oil. ^1H-NMR ($CDCl_3$) δ: 1.28 (3H, t, J=7.3 Hz), 1.41 (2H, m), 1.5—1.9 (4H, m), 1.9—2.2 (2H, m), 3.26 (2H, m), 3.56 (1H, m), 3.74 (3H, s), 4.13 (2H, m), 7.19 (1H, m). IR (neat) cm^{-1}: 3400—3200, 2950, 1730, 1650, 1560, 1450, 1370. HR-MS *m*/*z*: Calcd for $C_{13}H_{22}N_2O_5$: M, 286.1529. Found: 286.1539 (M^+).

Methyl N^{δ}-Phenyl-L-glutaminate (8c) A solution of **7c** (0.43 g) in TFA (10 ml) was stirred for 30 min at room temperature, then concentrated. The residue was dissolved in $CHCl_3$ and this solution was washed with 5% $NaHCO_3$ aqueous solution and water, successively. The organic layer was dried over Na_2SO_4, filtered, and concentrated, then the residue was chromatographed on silica gel with 5% MeOH in $CHCl_3$ to give **8c** (0.31 g, 98%) as a pale yellow syrup. ^1H-NMR ($CDCl_3$) δ: 1.8—2.0 (1H, m), 2.1—2.4 (1H, m), 2.5—2.6 (2H, m), 3.5—3.6 (1H, m), 3.74 (3H, s), 7.09 (1H, t, J=7.8 Hz), 7.31 (2H, t, J=7.8 Hz), 7.52 (2H, d, J=7.8 Hz), 8.23 (1H, s). IR (neat) cm^{-1}: 1732, 1666, 1600. HR-MS *m*/*z*: Calcd for $C_{12}H_{16}N_2O_3$: M, 236.1161. Found: 236.1123 (M^+).

Methyl N^{δ}-(3-Ethoxycarbonylphenyl)-L-glutaminate (8d) Using the same procedure as described for the preparation of **8c**, **8d** was prepared from **7d**. The yield of **8d** was 88%. Colorless oil. ^1H-NMR ($CDCl_3$) δ: 1.39 (3H, t, J=7.1 Hz), 1.8—2.0 (1H, m), 2.1—2.4 (1H, m), 2.5—2.7 (2H, m), 3.5—3.6 (1H, m), 3.74 (3H, s), 4.37 (2H, m), 7.39 (1H, t, J=7.8 Hz), 7.77 (1H, d, J=7.3 Hz), 7.94 (1H, d, J=7.8 Hz), 7.99 (1H, s), 8.63 (1H, s). IR (neat) cm^{-1}: 1718, 1610. HR-MS *m*/*z*: Calcd for

$C_{15}H_{20}N_2O_5$: M, 308.1372. Found: 308.1384 (M^+).

Methyl N^{δ}-Dimethyl-L-glutaminate (8e) Using the same procedure as described for the preparation of **8c**, **8e** was prepared from **7e**. The yield of **8e** was 98%. Pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 1.7—2.0 (1H, m), 2.0—2.3 (1H, m), 2.47 (2H, t, J=7.3 Hz), 2.95 (3H, s), 3.02 (3H, s), 3.5—3.6 (1H, m), 3.73 (3H, s). IR (neat) cm^{-1}: 1740, 1630. HR-MS m/z: Calcd for $C_8H_{16}N_2O_3$: M, 188.1161. Found: 188.1167 (M^+).

Methyl N^{α}-[(1-Benzyloxycarbonylindolin-5-yl)carbonyl]-N^{δ}-succinyl-L-ornithinate (10a) A mixture of **9** (653 mg) and DMF (30 ml) in $SOCl_2$ (3.0 ml) was stirred for 1 h and concentrated. The residue, together with K_2CO_3 (492 mg) and water (15 ml) was added to a solution of **8a** (730 mg) in CH_2Cl_2 (15 ml). The mixture was stirred for 10 h at room temperature, poured into 5% $NaHCO_3$ aqueous solution and extracted with $CHCl_3$. The organic layer was washed with 1 N HCl, dried over Na_2SO_4, filtered and concentrated. The residue was chromatographed on silica gel with 3% MeOH in $CHCl_3$ to give **10a** (590 mg, 54%) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 1.5—2.0 (4H, m), 2.67 (4H, s), 3.11 (2H, t, J=8.8 Hz), 3.52 (2H, m), 3.76 (3H, s), 4.07 (2H, t, J=8.8 Hz), 4.78 (1H, m), 5.26 (2H, s), 6.90 (1H, d, J=7.8 Hz), 7.2—7.5 (6H, m), 7.64 (2H, m). IR (neat) cm^{-1}: 3600—3200, 2950, 1770, 1650, 1610. HR-MS m/z: Calcd for $C_{27}H_{29}N_3O_7$: M, 507.2005. Found: 507.1996 (M^+).

Methyl N^{α}-[(1-Benzyloxycarbonylindolin-5-yl)carbonyl]-N^{δ}-[2-(ethoxycarbonyl)cyclopropylcarbonyl]-L-ornithinate (10b) Using the same procedure as described for the preparation of **10a**, **10b** was prepared from **8b**. The yield of **10b** was 57%. Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 1.29 (3H, m), 1.3—2.2 (8H, m), 3.16 (2H, m), 3.35 (2H, m), 3.79 (3H, s), 4.11 (4H, m), 4.79 (1H, m), 5.28 (2H, s), 6.27 (1H, m), 6.83 (1H, d, J=7.8 Hz), 7.39 (6H, m), 7.67 (2H, m). IR (neat) cm^{-1}: 3400—3300, 2960, 1760—1700, 1640, 1540. HR-MS m/z: Calcd for $C_{30}H_{35}N_3O_8$: M, 565.2424. Found: 565.2438 (M^+).

Methyl N^{α}-[(1-Benzyloxycarbonylindolin-5-yl)carbonyl]-N^{δ}-phenyl-L-glutaminate (10c) Using the same procedure as described for the preparation of **10a**, **10c** was prepared from **8c**. The yield of **10c** was 98%. Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 2.0—2.2 (1H, m), 2.3—2.6 (3H, m), 3.08 (2H, t, J=8.8 Hz), 3.77 (3H, s), 4.08 (2H, t, J=8.8 Hz), 4.7—4.9 (1H, m), 5.28 (2H, s), 7.07 (1H, t, J=7.3 Hz), 7.15 (1H, d, J=7.8 Hz), 7.28 (2H, m), 7.3—7.5 (5H, m), 7.58 (2H, d, J=7.8 Hz), 7.6—7.7 (2H, m), 7.86 (1H, s), 8.67 (1H, s). IR (neat) cm^{-1}: 1740, 1710, 1650, 1630. HR-MS m/z: Calcd for $C_{29}H_{29}N_3O_6$: M, 515.2056. Found: 515.2051 (M^+).

Methyl N^{α}-[(1-Benzyloxycarbonylindolin-5-yl)carbonyl]-N^{δ}-(3-ethoxycarbonylphenyl)-L-glutaminate (10d) Using the same procedure as described for the preparation of **10a**, **10d** was prepared from **8d**. The yield of **10d** was 86%. Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 1.37 (3H, t, J=7.1 Hz), 2.2—2.3 (1H, m), 2.3—2.6 (3H, m), 3.06 (2H, t, J=8.8 Hz), 3.77 (3H, s), 4.07 (2H, t, J=8.8 Hz), 4.3—4.4 (2H, m), 4.7—4.8 (1H, m), 5.28 (2H, s), 7.13 (1H, d, J=7.8 Hz), 7.3—7.5 (6H, m), 7.6—7.7 (3H, s), 7.74 (1H, d, J=7.8 Hz), 7.89 (1H, d, J=7.8 Hz), 8.12 (1H, s), 8.96 (1H, s). IR (neat) cm^{-1}: 1720, 1670, 1640, 1610. HR-MS m/z: Calcd for $C_{32}H_{33}N_3O_8$: M, 587.2268. Found: 587.2304 (M^+).

Methyl N^{α}-[(1-Benzyloxycarbonylindolin-5-yl)carbonyl]-N^{δ}-dimethyl-L-glutaminate (10e) Using the same procedure as described for the preparation of **10a**, **10e** was prepared from **8e**. The yield of **10e** was 15%. Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 2.2—2.3 (2H, m), 2.4—2.6 (2H, m), 2.94 (3H, s), 2.98 (3H, s), 3.15 (2H, t, J=8.8 Hz), 3.76 (3H, s), 4.10 (2H, t, J=8.8 Hz), 4.6—4.7 (1H, m), 5.28 (2H, s), 7.3—7.4 (7H, m), 7.71 (1H, s), 7.90 (1H, d, J=5.9 Hz). IR (neat) cm^{-1}: 1740, 1710, 1640, 1610. HR-MS m/z: Calcd for $C_{25}H_{29}N_3O_6$: M, 467.2056. Found: 467.2055 (M^+).

Methyl N^{α}-[1-[(2,4-Diaminopteridin-6-yl)methyl]indolin-5-ylcarbonyl]-N^{δ}-succinyl-L-ornithinate (12a) A mixture of **10a** (590 mg) and 5% Pd on carbon (120 mg) in MeOH (70 ml) was stirred under a hydrogen atmosphere overnight at room temperature and concentrated. The residue was dissolved with dimethylacetoamide (DMA, 2.0 ml), then **11** (126 mg) was added. The mixture was stirred at 55 °C for 6 h, poured into 5% $NaHCO_3$ aqueous solution, and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4, filtered and concentrated to afford a residue, which was chromatographed on silica gel with 10% MeOH in $CHCl_3$ to give **12a** (156 mg, 25%) as an orange powder. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 1.6—2.0 (4H, m), 2.71 (4H, s), 3.06 (2H, t, J=8.8 Hz), 3.55 (4H, m), 3.77 (3H, s), 4.52 (2H, s), 4.75 (1H, m), 6.52 (1H, d, J=7.8 Hz), 7.00 (1H, d, J=7.8 Hz), 7.58 (2H, m), 8.76 (1H, s). IR (KBr) cm^{-1}: 3000—3600, 2950, 1740, 1700, 1630, 1620, 1560, 1500, 1460. HR-FAB-MS m/z: Calcd for $C_{26}H_{30}N_9O_5$: MH, 548.2370. Found: 548.2379 (MH^+).

Methyl N^{α}-[1-[(2,4-Diaminopteridin-6-yl)methyl]indolin-5-ylcarbonyl]-N^{δ}-[2-(ethoxycarbonyl)cyclopropylcarbonyl]-L-ornithinate (12b) A solution of **10b** (1.0 g) in 30% HBr–CH_3COOH (10 ml) was stirred for 4 h at room temperature. The mixture was then poured into ether (100 ml) and the precipitated oil was washed by decantations with ether. It was then taken up in $CHCl_3$, washed with 5% $NaHCO_3$ aqueous solution, dried over Na_2SO_4, filtered and concentrated. The residue was redissolved in DMA (2.0 ml). To the resulting solution was added **11** (690 mg). The mixture was stirred at 55 °C for 6 h, poured into 5% $NaHCO_3$ aqueous solution, and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4, filtered and concentrated to afford a residue, which was chromatographed on silica gel with 10% MeOH in $CHCl_3$ to give **12b** (730 mg, 68%) as an orange powder. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 1.27 (3H, m), 1.3—2.2 (8H, m), 3.04 (2H, m), 3.38 (2H, m), 3.58 (2H, m), 3.78 (3H, s), 4.14 (2H, m), 4.54 (2H, s), 4.70 (1H, m), 6.54 (1H, d, J=8.3 Hz), 7.22 (1H, d, J=7.3 Hz), 7.45 (1H, m), 7.60 (2H, m), 8.77 (1H, s). IR (KBr) cm^{-1}: 3500—3200, 1730, 1630, 1450. HR-FAB-MS m/z: Calcd for $C_{29}H_{36}N_9O_6$: MH, 606.2789. Found: 606.2639 (MH^+).

Methyl N^{α}-[1-[(2,4-Diaminopteridin-6-yl)methyl]indolin-5-ylcarbonyl]-N^{δ}-phenyl-L-glutaminate (12c) Using the same procedure as described for the preparation of **12a**, **12c** was prepared from **10c** and **11**. The yield of **12c** was 31%. Orange powder. ^{1}H-NMR ($CDCl_3$) δ: 2.0—2.1 (1H, m), 2.4—2.5 (3H, m), 3.04 (2H, t, J=8.8 Hz), 3.58 (2H, t, J=8.8 Hz), 3.78 (3H, s), 4.53 (2H, s), 4.8—4.9 (1H, m), 6.48 (1H, d, J=7.8 Hz), 6.87 (1H, d, J=7.8 Hz), 7.08 (1H, t, J=7.8 Hz), 7.2—7.4 (2H, m), 7.6—7.7 (4H, m), 8.81 (1H, s). HR-FAB-MS m/z: Calcd for $C_{28}H_{30}N_9O_4$: MH, 556.2421. Found: 556.2564 (MH^+).

Methyl N^{α}-[1-[(2,4-Diaminopteridin-6-yl)methyl]indolin-5-ylcarbonyl]-N^{δ}-(3-ethoxycarbonylphenyl)-L-glutaminate (12d) Using the same procedure as described for the preparation of **12a**, **12d** was prepared from **10d** and **11**. The yield of **12d** was 18%. Orange powder. ^{1}H-NMR (DMSO-d_6) δ: 1.34 (3H, t, J=6.8 Hz), 1.9—2.4 (2H, m), 2.3—2.6 (2H, m), 3.00 (2H, t, J=7.3 Hz), 3.5—3.7 (5H, m), 4.32 (2H, m), 4.4—4.6 (3H, m), 6.6—6.7 (3H, s), 7.39 (1H, t, J=7.8 Hz), 7.6—7.7 (3H, m), 7.84 (1H, d, J=7.3 Hz), 8.22 (1H, s), 8.32 (1H, d, J=7.3 Hz), 8.73 (1H, s), 10.11 (1H, s). IR (KBr) cm^{-1}: 1710, 1630, 1610. HR-FAB-MS m/z: Calcd for $C_{31}H_{34}N_9O_6$: MH, 628.2632. Found: 628.2770 (MH^+).

Methyl N^{α}-[1-[(2,4-Diaminopteridin-6-yl)methyl]indolin-5-ylcarbonyl]-N^{δ}-dimethyl-L-glutaminate (12e) Using the same procedure as described for the preparation of **12a**, **12e** was prepared from **10e** and **11**. The yield of **12e** was 34%. Orange powder. ^{1}H-NMR (DMSO-d_6) δ: 1.9—2.2 (2H, m), 2.42 (2H, t, J=6.8 Hz), 2.84 (3H, s), 2.94 (3H, s), 3.00 (2H, t, J=8.3 Hz), 3.59 (2H, t, J=8.3 Hz), 3.64 (3H, s), 4.3—4.4 (1H, m), 4.55 (2H, s), 6.68 (1H, d, J=7.3 Hz), 7.6—7.7 (2H, m), 8.34 (1H, d, J=7.3 Hz), 8.71 (1H, s). IR (KBr) cm^{-1}: 1730, 1610. HR-FAB-MS m/z: Calcd for $C_{24}H_{30}N_9O_4$: MH, 508.2421. Found: 508.2389 (MH^+).

N^{δ}-Carboxypropionyl-N^{α}-[1-[(2,4-Diaminopteridin-6-yl)methyl]indolin-5-ylcarbonyl]-L-ornithine (3a) A solution of **12a** (154 mg) in EtOH (10 ml) was treated with 1 N NaOH (1.4 ml) and the mixture was stirred for 4 h at 35 °C, then concentrated. The residue was diluted to 10 ml with water and acidified to pH 3.7 with 1 N HCl. The resulting precipitated solid was collected by filtration and dried *in vacuo* to give **3a** (80 mg, 53%) as an orange powder. ^{1}H-NMR (DMSO-d_6) δ: 1.4—1.9 (4H, m), 2.32 (2H, m), 2.44 (2H, m), 3.05 (4H, m), 3.59 (2H, t, J=8.3 Hz), 4.36 (1H, m), 4.55 (2H, s), 6.68 (1H, m), 7.61 (2H, m), 7.80 (1H, m), 8.08 (1H, d, J=7.3 Hz), 8.72 (1H, s). IR (KBr) cm^{-1}: 3000—3500, 2940, 1710, 1640, 1600, 1550, 1500. FAB-MS m/z: 552 (MH^+). HR-FAB-MS m/z: Calcd for $C_{25}H_{30}N_9O_6$: MH, 552.2319. Found: 552.2355 (MH^+). mp 164—167 °C (dec.).

N^{δ}-(2-Carboxycyclopropylcarbonyl)-N^{α}-[1-[(2,4-Diaminopteridin-6-yl)methyl]indolin-5-ylcarbonyl]-L-ornithine (3b) Using the same procedure as described for the preparation of **3a**, **3b** was prepared from **12b**. The yield of **3b** was 67%. Orange powder. ^{1}H-NMR (DMSO-d_6) δ: 1.16 (2H, m), 1.4—1.9 (5H, m), 2.06 (1H, m), 3.04 (4H, m), 3.58 (2H, m), 4.37 (1H, m), 4.55 (2H, s), 6.69 (1H, d, J=8.3 Hz), 7.46 (1H, m), 7.62 (2H, m), 8.11 (1H, d, J=8.3 Hz), 8.72 (1H, s). IR (KBr) cm^{-1}: 3200—3500, 1640, 1610, 1500. FAB-MS m/z: 564 (MH^+). HR-FAB-MS m/z: Calcd for $C_{26}H_{30}N_9O_6$: MH, 564.2319. Found: 564.2371 (MH^+). mp 202—204 °C (dec.).

N^{α}-[1-[(2,4-Diaminopteridin-6-yl)methyl]indolin-5-ylcarbonyl]-N^{δ}-phenyl-L-glutamine (3c) Using the same procedure as described for the preparation of **3a**, **3c** was prepared from **12c**. The yield of **3c** was 77%. Orange powder. ^{1}H-NMR (DMSO-d_6) δ: 2.0—2.3 (2H, m), 2.4—2.5

(2H, m), 2.98 (2H, t, $J=7.8$ Hz), 3.59 (2H, t, $J=8.3$ Hz), 4.3—4.5 (1H, m), 4.56 (2H, s), 6.70 (1H, d, $J=7.8$ Hz), 6.90 (2H, s), 7.00 (1H, t, $J=7.8$ Hz), 7.26 (2H, t, $J=7.8$ Hz), 7.5—7.7 (4H, m), 7.7—7.9 (1H, m), 7.9—8.0 (1H, m), 8.20 (1H, d, $J=7.3$ Hz), 8.75 (1H, s), 9.93 (1H, s). IR (KBr) cm^{-1}: 1640. FAB-MS *m/z*: 542 (MH^+). HR-FAB-MS *m/z*: Calcd for $C_{27}H_{28}N_9O_4$: MH, 542.2264. Found: 542.2244 (MH^+). mp 208—210 °C (dec.).

N^{δ}-(3-Carboxyphenyl)-N^{α}-[1-[(2,4-Diaminopteridin-6-yl)methyl]indolin-5-ylcarbonyl]-L-glutamine (3d) Using the same procedure as described for the preparation of **3a**, **3d** was prepared from **12d**. The yield of **3d** was 84%. Orange powder. ^{1}H-NMR (DMSO-d_6) δ: 1.9—2.3 (2H, m), 2.4—2.6 (2H, m), 2.99 (2H, t, $J=8.8$ Hz), 3.59 (2H, t, $J=8.8$ Hz), 4.3—4.5 (1H, m), 4.59 (2H, s), 6.71 (1H, d, $J=8.3$ Hz), 6.93 (2H, s), 7.39 (1H, t, $J=7.8$ Hz), 7.5—7.8 (5H, m), 7.8—8.1 (1H, m), 8.1—8.2 (1H, m), 8.1—8.3 (2H, m), 8.75 (1H, s), 10.12 (1H, s). IR (KBr) cm^{-1}: 1638. FAB-MS *m/z*: 586 (MH^+). HR-FAB-MS *m/z*: Calcd for $C_{28}H_{28}N_9O_6$: MH, 586.2163. Found: 586.2262 (MH^+). mp 218—220 °C (dec.).

N^{α}-[1-[(2,4-Diaminopteridin-6-yl)methyl]indolin-5-ylcarbonyl]-N^{δ}-dimethyl-L-glutamine (3e) Using the same procedure as described for the preparation of **3a**, **3e** was prepared from **12e**. The yield of **3e** was 23%. Orange powder. ^{1}H-NMR (DMSO-d_6) δ: 1.9—2.1 (2H, m), 2.41 (2H, t, $J=7.1$ Hz), 2.82 (3H, s), 2.92 (3H, s), 3.01 (2H, t, $J=7.8$ Hz), 3.62 (2H, t, $J=7.8$ Hz), 4.2—4.4 (1H, m), 4.62 (2H, s), 6.72 (1H, d, $J=8.8$ Hz), 7.6—7.7 (2H, m), 8.24 (1H, d, $J=6.8$ Hz), 8.87 (1H, s). IR (KBr) cm^{-1}: 1726, 1640, 1610. FAB-MS *m/z*: 494 (MH^+). HR-FAB-MS *m/z*: Calcd for $C_{23}H_{28}N_9O_4$: MH, 494.2264. Found: 494.2293 (MH^+). mp 184—186 °C (dec.).

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