# Antirheumatic Agents. II.[1] Novel Methotrexate Derivatives Bearing an Alkyl-Substituted Benzene Ring

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**Novel methotrexate (MTX) derivatives with either a mono- or dialkyl-substituted benzene ring were synthesized and initially tested for *in vitro* anti-proliferative activities using human peripheral blood mononuclear cells (hPBMC) derived from healthy volunteers and synovial cells (hSC) derived from patients with rheumatoid arthritis (RA). Compounds with potent activities were further evaluated in an *in vivo* adjuvant arthritis model. In comparison with MTX, a glutamate derivative 3a was more potent as a suppressor of the *in vitro* cell proliferation and the *in vivo* experimental arthritis, and a homoglutamate derivative, 3e, exhibited fairly good activities *in vitro* and considerable activity *in vivo* in a dose-dependent manner. As expected, 3e did not act as a substrate of folylpolyglutamate synthetase (FPGS), and thus did not undergo polyglutamation, which is thought to be responsible for side-effects that occur during MTX therapy.**

**Key words** methotrexate; polyglutamation; antirheumatic activity; MX-33

We previously reported that MX-33, a methotrexate (MTX) derivative bearing an indoline moiety and homoglutamate instead of glutamate (Chart 1), had therapeutic advantages over its MTX predecessor for the treatment of rheumatoid arthritis (RA).[1] It potently inhibited the proliferation of human peripheral blood mononuclear cells (hPBMC) derived from healthy volunteers and synovial cells (hSC) derived from patients with RA and significantly suppressed progression of adjuvant arthritis in a rat model at the dose of 5.0 mg/kg (*p.o.*). Importantly, MX-33 did not act as a substrate for folylpolyglutamate synthetase (FPGS). Intracellularly, this enzyme catalyzes the formation of polyglutamates at the $\gamma$ position of glutamate of MTX,[2] and this process is thought to be responsible not only for the potentiation of the biological activity, but also for the associated hepatotoxic side effects.[3]

Consequently, we attempted to improve MX-33's antiarthritic potential by further modification of its aminobenzoic acid moiety, as well as investigation of the structure–activity relationships (SAR) of its derivatives. We were especially interested in comparing the biological profiles of derivatives containing an alkyl-substituted benzene ring with those of MX-33; these derivatives can be identical in shape to the ring-opened form of MX-33 at the indoline moiety. As regards the amino acid moiety, homoglutamate and glutamate were also compared to elucidate upon the SAR.

We report here the synthesis and the biological activities of novel MTX derivatives having an alkyl-substituted benzene ring (Chart 2).

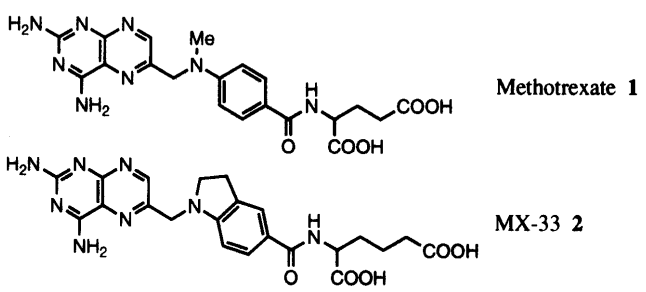
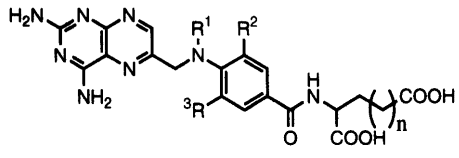



Chart 1. MTX 1 and MX-33 2

## Chemistry

Compounds **4a—c, e, f** were synthesized from commercially available materials as described in the experimental section. Compounds **3a—c, e, f** were synthesized from *N*-protected aminobenzoic acid forms of **4a—c, e, f** by the standard method shown in Chart 3. In brief, the compounds were converted into their acid chlorides by treatment with thionyl chloride in the presence of a catalytic amount of dimethylformamide (DMF). Subsequently, coupling with amino esters was performed by the Schotten-Baumann procedure to give the amides **5a—c, e, f**. The benzyloxycarbonyl group of **5a—c, e, f** was then removed by HBr-$CH_3COOH$ treatment or catalytic hydrogenation with palladium on carbon to yield the amines **6a—c, e, f**, which were effectively alkylated



| | $R^1$ | $R^2$ | $R^3$ | n |
|---|---|---|---|---|
| **3a** | Me | Me | H | 1 |
| **3b** | Et | Me | H | 1 |
| **3c** | Me | Me | Me | 1 |
| **3d** | H | Me | H | 1 |
| **3e** | Me | Me | H | 2 |
| **3f** | $-CH_2CH_2-$ | | Me | 2 |
| **3g** | H | Me | H | 2 |

Chart 2. Novel MTX Derivatives

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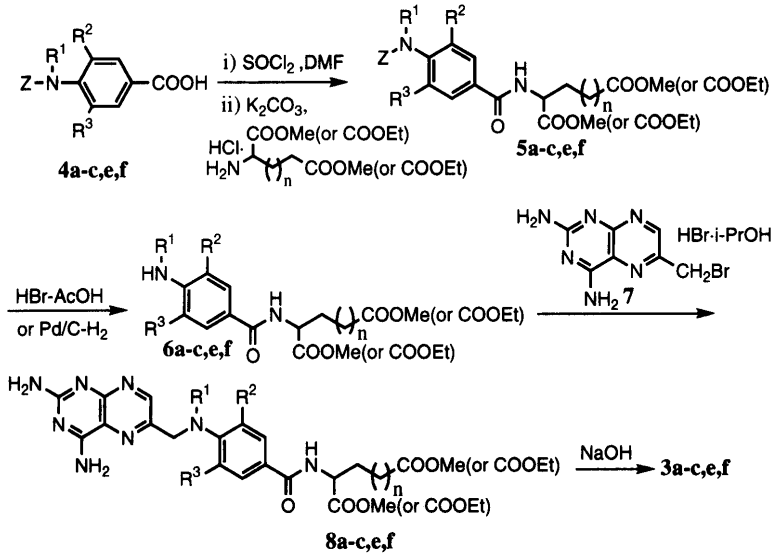


| | $R^1$ | $R^2$ | $R^3$ | n |
|---|---|---|---|---|
| **a series:** | Me | Me | H | 1 |
| **b series:** | Et | Me | H | 1 |
| **c series:** | Me | Me | Me | 1 |
| **d series:** | H | Me | H | 1 |
| **e series:** | Me | Me | H | 2 |
| **f series:** | $-CH_2CH_2-$ | | Me | 2 |
| **g series:** | H | Me | H | 2 |

($R^1$, $R^2$ and $R^3$ in this chart represent the same substituents as in Chart 2.)

Chart 3

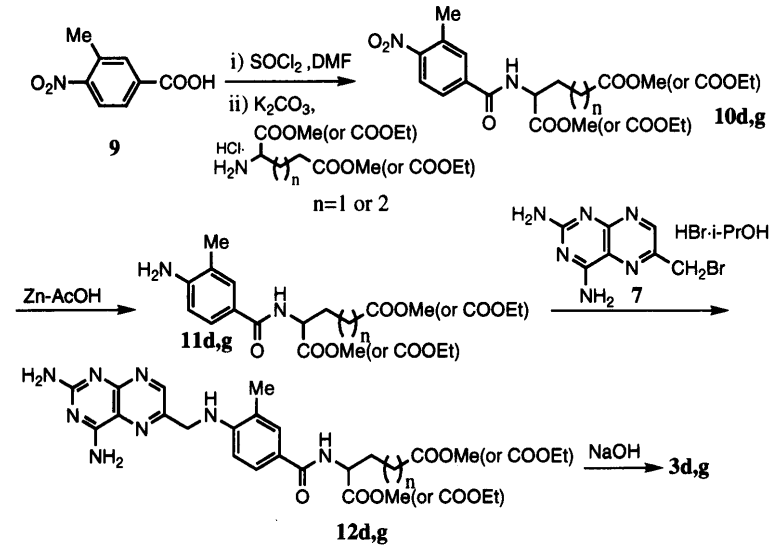


Chart 4

with 6-bromomethyl-2,4-diaminopteridine[4] **7** to produce **8a**—**c**, **e**, **f**. These compounds were next hydrolyzed with 1 N NaOH to give the final products **3a**—**c**, **e**, **f**.

Compounds **3d** and **3g** were synthesized from 3-methyl-4-nitrobenzoic acid **9** by the standard method with minor modifications, as shown in Chart 4. Compound **9** was converted into the amides **10d** and **10g** *via* the acid chloride using the same synthetic route as described above for **3a**—**c**, **e**, **f**. Compounds **10d** and **10g** were transformed into the corresponding amines **11d** and **11g** by treatment with zinc powder in acetic acid. These compounds were alkylated with **7** to produce the diesters **12d** and **12g**, which were finally hydrolyzed with 1 N NaOH to yield **3d** and **3g**.

## Results and Discussion

Table 1 shows that all the compounds tested *in vitro* inhibited cell proliferation, which occurs during the course

Table 1. Anti-proliferative Activities of Novel MTX Derivatives

| Compound No. | $IC_{50}$ (nM) values | |
|---|---|---|
| | Human PBMC[a] | Human SC[b] |
| **3a** | 4.6 | 14 |
| **3b** | 5.0 | 35 |
| **3c** | 5.9 | 41 |
| **3d** | 2.1 | n.d.[c] |
| **3e** | 13 | 31 |
| **3f** | 53 | n.d.[c] |
| **3g** | 77 | n.d.[c] |
| MX-33 **2** | 20 | 230 |
| MTX | 24 | 61 |

*a*) PBMC ($1 \times 10^5$ cells) from several healthy donors were cultured with several concentrations of drugs and PHA (0.3 $\mu$g/ml) for 3 d. [$^3$H]UdR (1 $\mu$Ci/well) was added to each well for the last 5 h of culture and the proliferation was assessed by determining [$^3$H]UdR uptake into the cells. The results are the mean values of triplicate assays. *b*) SC ($3 \times 10^3$ cells) from synovial membrane of RA patients were cultured with several concentrations of drugs for 5 d. [$^3$H]UdR was added for the last 2 d of culture and the proliferation was assessed by determining [$^3$H]UdR uptake into the cells. The results are the mean of triplicate assays. *c*) Not determined.

Table 2. Activities of MTX Derivatives as Substrates of FPGS

| Compound No. | Drug concentration ($\mu$M) | FPGS activity (nmol/mg/h) |
|---|---|---|
| MTX | 100 | 2.64 |
| **3e** | 100 | 0.0864 |
| **3e** | 500 | 0.0957 |

FPGS activity was determined as described in ref. 5, with partially purified enzyme from rat liver. The results are the mean values of triplicate assays.

of synovial inflammation. Inhibition of hPBMC proliferation by the glutamate derivative **3d** was 12 times more potent than that by MTX with an $IC_{50}$ value of 2.1 nM, while under similar conditions, **3a**, **3b** and **3c** were 5, 5 and 4 times more potent than MTX, respectively, with $IC_{50}$ values of 4.6, 5.0 and 5.9 nM. In the hSC proliferative assay, **3a** also showed a strong anti-proliferative activity with the $IC_{50}$ of 14 nM, whereas that of MTX was 61 nM. Inhibitions of hSC proliferation by **3b** and **3c** were comparable to that by MTX.

The anti-proliferative effect of the homoglutamate **3e**, derived by modification of the amino acid moiety of **3a**, was slightly greater than that of MTX on hPBMC and considerably greater than that of MX-33 on hSC. The greater anti-proliferative effect of **3e** compared to MTX on hPBMC and hSC is presumably attributable to the alkyl group substitution on the benzene ring.

Glutamate derivatives (**3a, d**) were more potent than their homoglutamate counterparts (**3e, g**) in the inhibition of hPBMC proliferation (Table 1). These results support the concept that polyglutamation can only occur in derivatives with a COOH group at the $\gamma$ position. To provide further support for this concept, we used Moran's method[5] to show the presence and absence of substrate activity for FPGS by MTX and **3e**, respectively (Table 2).

Antirheumatic activities of **3a** and **3e** were assessed *in vivo* by using rat adjuvant arthritis model.[6] Compound **3a** completely suppressed the development of arthritis at a *p.o.* dose of 0.25 mg/kg; a similar suppression was observed with MTX at the same dose (Table 3). In addition, dose-dependent suppression was observed after oral administration of **3e** at doses ranging from 2.5 to 40 mg/kg.

Table 3. Effects of **3a**, **3e** and MTX on the Development of Adjuvant Arthritis in Rats

| Compound | Mean arthritis score (% suppression) |
|---|---|
| MTX 0.25 mg/kg | 12 ($p<0.02$) |
| **3a** 0.10 mg/kg | 39 ($p<0.02$) |
| **3a** 0.25 mg/kg | 8 ($p<0.02$) |
| **3e** 2.50 mg/kg | 93 (N.S.) |
| **3e** 10.0 mg/kg | 54 (N.S.) |
| **3e** 40.0 mg/kg | 2 ($p<0.02$) |

Drug were suspended in 0.1% CMC-Na solution and administered orally 5 times a week for 2 weeks from the day of adjuvant injection. Each group consisted of 5 rats (Lewis, 6-week-old). Arthritic score was evaluated on day 21, when it was maximum in the control group. The percentage suppression was expressed as follows:

$$\% \text{ suppression} = \{(\text{control group} - \text{treated group})/\text{control group}\} \times 100$$

Statistical significance of differences from the control were analyzed by means of Wilcoxon's rank sum test.

As polyglutamation causes side-effects, but is also important for the efficacy of MTX, derivatives which can not undergo this process must achieve sufficient *in vivo* pharmacological effects by some other mechanism. In this respect, the homoglutamate derivative **3e**, found to be dose-dependently, orally active against rat adjuvant arthritis, can be regarded as a significant lead compound for future development.

## Experimental

$^1$H-NMR spectra were recorded on a JEOL model JMN-FX200 NMR spectrometer with $Me_4Si$ as the reference. Infrared spectra were run on a Hitachi 270-3 infrared spectrometer. Mass spectra were run on a Shimadzu GCMS-QP1000, FAB and HR-FAB mass spectra on a VG Analytical VG11-250, and HR mass spectra on a Fisons OPUS-3100. TLC was routinely performed on Merck Kieselgel 60 F254. HPLC analyses were performed with a Hitachi L-3000 detector, Hitachi L-6200 pump, and YMC-Pack A-312 S-5 120A ODS column. Melting points were taken on a Yanaco Model MP.

***N*-Benzyloxycarbonyl-4-amino-3-methylbenzoic Acid** To a suspension of 4-amino-3-methylbenzoic acid (5.0 g) in water (80 ml) were added a solution of benzyloxycarbonyl chloride (3.7 g) in ether (20 ml) and $NaHCO_3$ (3.6 g) at 0 °C, and the mixture was stirred vigorously for 3 h at room temperature. Furthermore, to the mixture were added an additional benzyloxycarbonyl chloride (3.7 g) in ether (95 ml) and $NaHCO_3$ (7.2 g) at 0 °C and this mixture was stirred vigorously at room temperature for 2 h. The mixture was acidified with 4 N HCl and the resulting precipitates were collected by filtration, washed with water and hexane successively, and dried under vacuum to give *N*-benzyloxycarbonyl-4-amino-3-methylbenzoic acid (2.8 g, 30%) as a white solid. $^1$H-NMR ($CDCl_3$) $\delta$: 2.38 (3H, s), 5.20 (2H, s), 7.41 (6H, m), 7.97 (3H, m). IR (KBr) $cm^{-1}$: 3320, 3100—2800, 1690, 1610. MS *m/z*: 285 ($M^+$), 268, 151, 92, 91.

**Methyl *N*-Benzyloxycarbonyl-3-methyl-4-methylaminobenzoate** To a suspension of NaH (2.3 g, 60%) in dimethylformamide (DMF, 10 ml) was added a solution of *N*-benzyloxycarbonyl-4-amino-3-methylbenzoic acid (2.8 g) in DMF (10 ml) at 0 °C and the mixture was stirred for 15 min at the same temperature. To this mixture was added MeI (4 ml) and the resulting mixture was stirred for 2 h at room temperature, poured into water, and extracted with toluene. The organic layer was washed with brine, dried over $Na_2SO_4$, filtered and concentrated under reduced pressure. The residue was chromatographed on silica gel with $CHCl_3$ to give methyl *N*-benzyloxycarbonyl-3-methyl-4-methylaminobenzoate (1.8 g, 59%) as a colorless oil. $^1$H-NMR ($CDCl_3$) $\delta$: 2.19 (3H, s), 3.19

(3H, s), 3.87 (3H, s), 5.09 (2H, s), 7.21 (6H, m), 7.85 (2H, m). IR (KBr) $cm^{-1}$: 2950, 1720, 1610, 1580. MS *m/z*: 313 ($M^+$), 295, 177, 92, 91. HR-MS *m/z*: Calcd for $C_{18}H_{19}NO_4$: M, 313.1314. Found: 313.1310 ($M^+$).

***N*-Benzyloxycarbonyl-3-methyl-4-methylaminobenzoic Acid (4a)** To a solution of methyl *N*-benzyloxycarbonyl-3-methyl-4-aminomethylbenzoate (1.8 g) in EtOH (15 ml) was added a 2 N NaOH solution (15 ml) and the mixture was refluxed for 2 h, then concentrated to 15 ml under reduced pressure. The residual solution was acidified with 4 N HCl, and extracted with $CHCl_3$. The organic solution was dried over $Na_2SO_4$, filtered and concentrated to give **4a** (1.5 g, 87%) as a white powder. $^1$H-NMR ($CDCl_3$) $\delta$: 2.02 (3H, s), 3.04 (3H, s), 4.69 (2H, s), 7.08 (6H, m), 7.77 (2H, m). IR (KBr) $cm^{-1}$: 3660, 3460, 1710, 1690. MS *m/z*: 299 ($M^+$), 158. HR-MS *m/z*: Calcd for $C_{17}H_{17}NO_4$: M, 299.1158. Found: 299.1163 ($M^+$).

**Diethyl *N*-(4-(*N'*-Benzyloxycarbonyl-*N'*-methylamino)-3-methylbenzoyl)-L-glutamate (5a)** A mixture of **4a** (820 mg) and DMF (0.2 ml) in $SOCl_2$ (5 ml) was stirred for 2 h at room temperature and then concentrated under reduced pressure. To a solution of the acid chloride synthesized above in $CH_2Cl_2$ (20 ml) were added diethyl glutamate hydrochloride (700 mg), $K_2CO_3$ (1.4 g) and water (20 ml). The mixture was stirred vigorously for 12 h at room temperature, poured into water and extracted with $CHCl_3$. The organic layer was washed with 1 N HCl, dried, concentrated and chromatographed on silica gel with $CHCl_3$–MeOH (100 : 3) to give **5a** (700 mg, 53%) as a colorless oil. $^1$H-NMR ($CDCl_3$) $\delta$: 1.1—1.5 (6H, m), 2.0—2.7 (4H, m), 2.18 (3H, s), 3.19 (3H, s), 3.9—4.4 (4H, m), 4.80 (1H, m), 5.10 (2H, s), 7.0—7.5 (7H, m), 7.63 (2H, m). IR (neat) $cm^{-1}$: 3400—3300, 2980, 1740, 1710, 1660. MS *m/z*: 484 ($M^+$), 411, 282, 91. HR-MS *m/z*: Calcd for $C_{25}H_{30}N_2O_7$: M, 484.2210. Found: 484.2176 ($M^+$).

**Diethyl *N*-(4-(*N'*-Methylamino)-3-methylbenzoyl)-L-glutamate (6a)** A mixture of **5a** (700 mg) and anisole (700 mg) in 30% HBr-$CH_3COOH$ (7 ml) was stirred for 4 h at room temperature. Ether (200 ml) was added to the reaction mixture and the resulting precipitates were collected, washed with ether by decantation, and suspended in $CHCl_3$ (10 ml). To this suspension was added 5% $NaHCO_3$ solution, and the mixture was stirred vigorously. The organic layer was separated, dried, and concentrated to afford pure **6a** (360 mg, 71%) as a colorless oil. $^1$H-NMR ($CDCl_3$) $\delta$: 1.0—1.5 (6H, m), 2.11 (3H, s), 2.1—2.7 (4H, m), 2.90 (3H, s), 3.9—4.4 (4H, m), 4.80 (1H, m), 6.54 (1H, d, $J$=8 Hz), 6.80 (1H, d, $J$=7 Hz), 7.61 (2H, m). IR (neat) $cm^{-1}$: 3400, 2980, 1740, 1640, 1610. MS *m/z*: 350 ($M^+$), 164, 149, 148.

**Diethyl *N*-[4-(*N'*-(2,4-Diaminopteridin-6-ylmethyl)-*N'*-methylamino)-3-methylbenzoyl]-L-glutamate (8a)** A mixture of **6a** (360 mg) and 6-bromomethyl-2,4-diaminopteridine·HBr·iso-PrOH (**7**, 407 mg) in dimethylacetoamide (DMA, 6 ml) was stirred at 55—65 °C for 4 h and then cooled to room temperature. The mixture was poured into 5% $NaHCO_3$ solution and extracted with $CHCl_3$. The organic layer was dried, concentrated and chromatographed on silica gel with $CHCl_3$–MeOH (10 : 1) to give **8a** (280 mg, 52%) as a yellow powder. $^1$H-NMR ($CDCl_3$–$CD_3OD$) $\delta$: 1.2—1.4 (6H, m), 2.0—2.6 (4H, m), 2.41 (3H, s), 2.73 (3H, s), 4.0—4.3 (4H, m), 4.32 (2H, s), 4.76 (1H, m), 7.06 (1H, d, $J$=8.3 Hz), 7.41 (1H, d, $J$=7.8 Hz), 7.65 (2H, m), 8.79 (1H, s). IR (KBr) $cm^{-1}$: 3400—3300, 3000, 1740, 1640. MS *m/z*: 524 ($M^+$), 498, 322, 176. HR-FAB-MS *m/z*: Calcd for $C_{25}H_{33}N_8O_5$: MH, 525.2584. Found: 525.2574 ($MH^+$).

***N*-[4-(*N'*-(2,4-Diaminopteridin-6-ylmethyl)-*N'*-methylamino)-3-methylbenzoyl]-L-glutamic Acid (3a)** To a solution of **8a** (280 mg) in EtOH (20 ml) was added 1 N NaOH (1.6 ml) and the mixture was stirred overnight at room temperature then concentrated under reduced pressure. The obtained residue was dissolved in water (10 ml) and acidified to pH 3.7 with 1 N HCl. The resulting precipitate was collected by filtration and dried to give **3a** (180 mg, 73%) as a yellow powder. $^1$H-NMR (DMSO-$d_6$) $\delta$: 1.9—2.2 (2H, m), 2.35 (2H, m), 2.42 (3H, s), 2.73 (3H, s), 4.34 (2H, s), 4.41 (1H, m), 7.10 (1H, d, $J$=8.3 Hz), 7.72 (2H, m), 8.38 (1H, d, $J$=7.8 Hz), 8.65 (1H, s). IR (KBr) $cm^{-1}$: 3400—3100, 1640, 1560, 1490. FAB-MS *m/z*: 469 ($MH^+$). mp 181—184 °C (dec.). Analysis by HPLC (solvent, $CH_3COOH$/$CH_3COONa$, pH 5.4: MeOH = 4 : 1; flow rate, 1.0 $cm^3$/min; detection, 254 nm) showed the purity to be at least 98% (retention time 19 min).

**Ethyl *N*-Benzyloxycarbonyl-4-ethylamino-3-methylbenzoate** Using the same procedure as described for the preparation of methyl *N*-benzyloxycarbonyl-3-methyl-4-methylaminobenzoate, ethyl *N*-benzyloxycarbonyl-4-ethylamino-3-methylbenzoate was prepared from ethyl 4-ethylamino-3-methylbenzoate and benzyloxycarbonyl chloride. The yield of ethyl *N*-benzyloxycarbonyl-4-ethylamino-3-methylbenzoate was 87%. Colorless oil. $^1$H-NMR ($CDCl_3$) $\delta$: 0.9—1.7 (6H, m), 2.16 (3H, s), 3.3—3.9 (2H, m), 4.1—4.6 (2H, m), 5.07 (2H, s), 6.9—7.4 (6H, m), 7.6—8.0 (2H, m). IR (neat) $cm^{-1}$: 2980, 1710, 1610. MS *m/z*: 341 ($M^+$).

**4-(*N*-Benzyloxycarbonyl-*N*-ethylamino)-3-methylbenzoic Acid (4b)** Using the same procedure as described for the preparation of **4a**, **4b** was prepared from ethyl *N*-benzyloxycarbonyl-4-ethylamino-3-methylbenzoate. The yield of **4b** was 87%. White powder. $^1$H-NMR ($CDCl_3$) $\delta$: 1.16 (3H, t, $J$=5.4 Hz), 2.20 (3H, s), 3.3—4.0 (2H, m), 5.12 (2H, s), 6.9—7.5 (6H, m), 7.7—8.1 (2H, m). IR (KBr) $cm^{-1}$: 2970, 1710, 1610. MS *m/z*: 313 ($M^+$), 173, 91. HR-MS *m/z*: Calcd for $C_{18}H_{19}NO_4$: M, 313.1314. Found: 313.1300 ($M^+$).

**Diethyl *N*-(4-(*N'*-Benzyloxycarbonyl-*N'*-ethylamino)-3-methylbenzoyl)-L-glutamate (5b)** Using the same procedure as described for the preparation of **5a**, **5b** was prepared from **4b** and diethyl glutamate·hydrochloride. The yield of **5b** was 99%. Colorless oil. $^1$H-NMR ($CDCl_3$) $\delta$: 0.9—1.5 (9H, m), 1.9—2.7 (7H, m), 3.3—3.6 (2H, m), 3.6—4.5 (4H, m), 4.5—5.0 (1H, m), 5.08 (2H, s), 6.9—8.2 (9H, m). IR (neat) $cm^{-1}$: 3350, 2980, 1710, 1610, 1580. MS *m/z*: 498 ($M^+$), 296, 91.

**Diethyl *N*-(3-Methyl-4-Ethylaminobenzoyl)-L-glutamate (6b)** Using the same procedure as described for the preparation of **6a**, **6b** was prepared from **5b**. The yield of **6b** was 59%. Pale brown oil. $^1$H-NMR ($CDCl_3$) $\delta$: 1.0—1.5 (9H, m), 1.8—2.7 (7H, m), 2.9—3.5 (2H, m), 3.8—4.4 (4H, m), 4.5—5.0 (1H, m), 6.3—7.0 (2H, m), 7.3—7.8 (2H, m). IR (neat) $cm^{-1}$: 3380, 2980, 1730, 1640, 1610. MS *m/z*: 363 ($M^+$), 162.

**Diethyl *N*-[4-(*N'*-(2,4-Diaminopteridin-6-ylmethyl)-*N'*-ethylamino)-3-methylbenzoyl]-L-glutamate (8b)** Using the same procedure as described for the preparation of **8a**, **8b** was prepared from **6b** and **7**. The yield of **8b** was 35%. Yellow powder. $^1$H-NMR ($CDCl_3$) $\delta$: 1.06 (3H, t, $J$=6.8 Hz), 1.1—1.5 (6H, m), 1.76 (3H, s), 2.3—2.6 (4H, m), 3.08 (2H, m), 4.10 (2H, m), 4.38 (2H, m), 4.7—4.9 (1H, m), 5.32 (2H, s), 6.94 (1H, d, $J$=7.4 Hz), 7.05 (1H, d, $J$=8.8 Hz), 7.56 (1H, d, $J$=5.2 Hz), 7.67 (1H, s), 8.80 (1H, s). IR (KBr) $cm^{-1}$: 3500—3300, 3140, 1980, 1730, 1630. MS *m/z*: 538 ($M^+$), 363, 175. HR-FAB-MS *m/z*: Calcd for $C_{26}H_{35}N_8O_5$: MH, 539.2730. Found: 539.2731 ($MH^+$).

***N*-[4-(*N'*-(2,4-Diaminopteridin-6-ylmethyl)-*N'*-ethylamino)-3-methylbenzoyl]-L-glutamic Acid (3b)** Using the same procedure as described for the preparation of **3a**, **3b** was prepared from **8b**. The yield of **3c** was 89%. Yellow powder. $^1$H-NMR (DMSO-$d_6$) $\delta$: 1.04 (3H, t, $J$=6.8 Hz), 1.8—2.3 (2H, m), 2.3—2.5 (5H, m), 3.04 (2H, m), 4.39 (3H, m), 7.10 (1H, d, $J$=8.3 Hz), 7.61 (1H, d, $J$=8.3 Hz), 7.72 (1H, s), 8.34 (1H, d, $J$=7.8 Hz), 8.57 (1H, s). IR (KBr) $cm^{-1}$: 3350, 2350, 1630, 1490. FAB-MS *m/z*: 483 ($MH^+$). mp 175—178 °C (dec.). Analysis by HPLC (solvent, $CH_3COOH$/$CH_3COONa$, pH 5.4: MeOH = 4 : 1; flow rate, 1.0 $cm^3$/min; detection, 254 nm) showed the purity to be at least 98% (retention time 51 min).

**Ethyl *N*-Benzyloxycarbonyl-4-amino-3,5-dimethylbenzoate** To a suspension of NaH (0.82 g, 60% in mineral oil) in tetrahydrofuran (THF, 100 ml) was added ethyl 4-amino-3,5-dimethylbenzoate[8] (2.0 g) and the mixture was stirred for 30 min at room temperature. To this mixture was added benzyloxycarbonyl chloride (4.4 ml) and the resulting mixture was stirred overnight, poured into ice-water, and extracted with AcOEt. The organic layer was washed with brine, dried over $Na_2SO_4$ and concentrated. The residual solids were suspended in hexane and the suspension was filtered. The obtained solid was dried under vacuum to give ethyl *N*-benzyloxycarbonyl-4-amino-3,5-dimethylbenzoate (3.2 g, 95%) as a white powder. $^1$H-NMR ($CDCl_3$) $\delta$: 1.39 (3H, t, $J$=7 Hz), 2.05 (6H, s), 4.35 (2H, m), 5.15 (2H, s), 7.23 (6H, m), 7.75 (2H, s). IR (neat) $cm^{-1}$: 2980, 1790, 1720, 1610, 1500. MS *m/z*: 327 ($M^+$), 220, 174, 91. HR-MS *m/z*: Calcd for $C_{19}H_{21}NO_4$: M, 327.1471. Found: 327.1430 ($M^+$).

**Ethyl *N*-Benzyloxycarbonyl-4-methylamino-3,5-dimethylbenzoate** To a suspension of NaH (1.2 g, 60% in mineral oil) was added *N*-benzyloxycarbonyl-4-amino-3,5-dimethylbenzoate (3.2 g) and the mixture was stirred for 15 min. To this mixture was added MeI (1.8 ml) and the resulting mixture was stirred overnight at room temperature, poured into ice-water, and extracted with AcOEt. The organic layer was washed with brine, dried over $Na_2SO_4$ and concentrated under reduced pressure. The residue was chromatographed on silica gel with hexane–AcOEt (10 : 1) to give ethyl *N*-benzyloxycarbonyl-4-methylamino-3,5-dimethylbenzoate (1.47 g, 44%) as a colorless oil. $^1$H-NMR ($CDCl_3$) $\delta$: 1.38 (3H, t, $J$=7 Hz), 2.21 (6H, s), 3.17 (3H, s), 4.42 (2H, m), 5.13 (2H, s), 7.0—7.6 (5H, m), 7.80 (2H, s). IR (neat) $cm^{-1}$: 2960, 1700, 1600, 1490. MS *m/z*:

341 ($M^+$), 91. HR-MS *m/z*: Calcd for $C_{20}H_{23}NO_4$: M, 341.1627. Found: 341.1667 ($M^+$).

***N*-Benzyloxycarbonyl-4-methylamino-3,5-dimethylbenzoic Acid (4c)** Using the same procedure as described for the preparation of **4a**, **4c** was prepared from ethyl *N*-benzyloxycarbonyl-4-methylamino-3,5-dimethylbenzoate. The yield of **4c** was 99%. Colorless oil. $^1$H-NMR ($CDCl_3$) $\delta$: 2.18 (6H, s), 3.14 (3H, s), 5.04 (2H, s), 6.9—7.5 (5H, m), 7.76 (2H, s). IR (neat) $cm^{-1}$: 3600—3300, 2950, 1710, 1610. MS *m/z*: 313 ($M^+$), 91. HR-MS *m/z*: Calcd for $C_{18}H_{19}NO_4$: M, 313.1314. Found: 313.1313 ($M^+$).

**Diethyl *N*-(4-(*N'*-Benzyloxycarbonyl-*N'*-methylamino)-3,5-dimethylbenzoyl)-L-glutamate (5c)** Using the same procedure as described for the preparation of **5a**, **5c** was prepared from **4c**. The yield of **5c** was 99%. Colorless oil. $^1$H-NMR ($CDCl_3$) $\delta$: 1.23 (3H, t, $J$=7 Hz), 1.31 (3H, t, $J$=7 Hz), 2.1—2.7 (4H, m), 2.17 (6H, s), 3.13 (3H, s), 4.13 (2H, m), 4.25 (2H, m), 4.73 (1H, m), 5.07 (2H, s), 6.94 (1H, d, $J$=7 Hz), 7.1—7.5 (5H, m), 7.51 (2H, s). IR (neat) $cm^{-1}$: 3350, 2980, 1730, 1720, 1710, 1690, 1650. MS *m/z*: 498 ($M^+$), 296. HR-MS *m/z*: Calcd for $C_{26}H_{32}N_2O_7$: M, 498.2366. Found: 498.2339 ($M^+$).

**Diethyl *N*-(4-Methylamino-3,5-dimethylbenzoyl)-L-glutamate (6c)** Using the same procedure as described for the preparation of **6a**, **6c** was prepared from **5c**. The yield of **6c** was 77%. Brown oil. $^1$H-NMR ($CDCl_3$) $\delta$: 1.21 (3H, t, $J$=7 Hz), 1.29 (3H, t, $J$=7 Hz), 2.0—2.8 (4H, m), 2.29 (6H, s), 2.87 (3H, s), 3.11 (1H, s), 4.11 (2H, m), 4.23 (2H, m), 4.83 (1H, m), 6.83 (1H, d, $J$=8 Hz), 7.43 (2H, s). MS *m/z*: 364 ($M^+$), 162.

**Diethyl *N*-[4-(*N'*-(2,4-Diaminopteridin-6-ylmethyl)-*N'*-methylamino-3,5-dimethylbenzoyl]-L-glutamate (8c)** Using the same procedure as described for the preparation of **8a**, **8c** was prepared from **6c** and **7**. The yield of **8c** was 40%. Yellow powder. $^1$H-NMR ($CDCl_3$) $\delta$: 1.25 (3H, t, $J$=7 Hz), 1.32 (3H, t, $J$=7 Hz), 2.0—2.7 (4H, m), 2.42 (6H, s), 2.82 (3H, s), 4.18 (2H, m), 4.28 (2H, m), 4.42 (2H, s), 4.78 (1H, m), 7.38 (1H, d, $J$=7.3 Hz), 7.53 (2H, s), 8.89 (1H, s). IR (KBr) $cm^{-1}$: 3500—3300, 1740, 1630, 1560. MS *m/z*: 538 ($M^+$), 506, 207, 176. HR-FAB-MS *m/z*: Calcd for $C_{26}H_{35}N_8O_5$: MH, 539.2730. Found: 539.2728 ($MH^+$).

***N*-[4-(*N'*-(2,4-Diaminopteridin-6-ylmethyl)-*N'*-methylamino)-3,5-dimethylbenzoyl]-L-glutamic Acid (3c)** Using the same procedure as described for the preparation of **3a**, **3c** was prepared from **8c**. The yield of **3c** was 89%. Yellow powder. $^1$H-NMR (DMSO-$d_6$) $\delta$: 1.8—2.2 (2H, m), 2.3—2.5 (2H, m), 2.34 (6H, s), 2.73 (3H, s), 4.3—4.5 (1H, m), 4.36 (2H, s), 7.55 (2H, s), 8.40 (1H, d, $J$=6 Hz), 8.74 (1H, s). IR (KBr) $cm^{-1}$: 3400—2900, 1650, 1540, 1480, 1230. FAB-MS *m/z*: 483 ($MH^+$). mp 215—218 °C (dec.). Analysis by HPLC (solvent, $CH_3COOH/CH_3COONa$, pH 5.4 : MeOH = 4 : 1; flow rate, 1.0 $cm^3$/min; detection, 254 nm) showed the purity to be at least 98% (retention time 71 min).

**Diethyl *N*-(3-Methyl-4-nitrobenzoyl)-L-glutamate (10d)** A mixture of 4-nitro-3-methylbenzoic acid (10 g) and DMF (20 $\mu$l) in $SOCl_2$ (30 ml) was refluxed for 2 h and the mixture was cooled to room temperature, then concentrated under reduced pressure. The residue was dissolved in $CH_2Cl_2$ (250 ml). To this solution were added diethyl glutamate hydrochloride (13.4 g), $K_2CO_3$ (30 g) and water (250 ml). The reaction mixture was stirred vigorously for 12 h, then poured into water and extracted with $CHCl_3$. The organic layer was dried over $Na_2SO_4$ and concentrated under reduced pressure. The residue was chromatographed on silica gel with $CHCl_3$–MeOH (100 : 1) to give **10d** (10 g, 49%) as a colorless oil. $^1$H-NMR ($CDCl_3$) $\delta$: 1.2—1.4 (6H, m), 2.0—2.4 (2H, m), 2.4—2.6 (2H, m), 2.62 (3H, s), 4.14 (2H, m), 4.25 (2H, m), 4.76 (1H, m), 7.44 (1H, d, $J$=7.3 Hz), 7.76 (1H, m), 7.81 (1H, s), 7.98 (1H, d, $J$=8.3 Hz). IR (neat) $cm^{-1}$: 3350, 3000, 1740, 1530. MS *m/z*: 366 ($M^+$), 321, 293, 164.

**Diethyl *N*-(4-Amino-3-methylbenzoyl)-L-glutamate (11d)** To a solution of **10d** (10 g) was added zinc powder (18 g) at 0 °C and the mixture was stirred for 2 h at room temperature. The reaction mixture was filtered and the filtrate was concentrated under reduced pressure. The obtained residue was diluted to 200 ml with $CHCl_3$. The organic layer was washed with 5% $NaHCO_3$ solution, dried over $Na_2SO_4$ and concentrated under reduced pressure to give **11d** (7.8 g, 85%) as a colorless oil. $^1$H-NMR ($CDCl_3$) $\delta$: 1.1—1.4 (6H, m), 2.15 (3H, s), 2.0—2.4 (4H, m), 4.0—4.3 (4H, m), 4.77 (1H, m), 6.62 (1H, d, $J$=8.3 Hz), 6.81 (1H, d, $J$=7.3 Hz), 7.4—7.6 (2H, m). IR (neat) $cm^{-1}$: 3370, 3000, 1740, 1630. MS *m/z*: 336 ($M^+$), 290, 77.

**Diethyl *N*-[4-(*N'*-(2,4-Diaminopteridin-6-ylmethyl))amino-3-methylbenzoyl]-L-glutamate (12d)** A suspension of **11d** (1.9 g) and **7** (1.0 g) in DMA (16 ml) was stirred for 5 h at 55—65 °C. The reaction mixture was poured into 5% $NaHCO_3$ solution and extracted with $CHCl_3$–MeOH (1 : 1). The organic layer was dried over $Na_2SO_4$ and concentrated under reduced pressure. The residue was chromatographed on silica gel with $CHCl_3$–MeOH (10 : 1) to give **12d** (580 mg, 20%) as a yellow amorphous solid. $^1$H-NMR ($CDCl_3$–$CD_3OD$) $\delta$: 1.2—1.4 (6H, m), 2.30 (3H, s), 2.0—2.5 (4H, m), 4.0—4.3 (4H, m), 4.67 (3H, m), 6.61 (1H, d, $J$=7.8 Hz), 7.59 (3H, m), 8.75 (1H, s). IR (KBr) $cm^{-1}$: 3500—3300, 1730, 1630, 1610. HR-FAB-MS *m/z*: Calcd for $C_{24}H_{31}N_8O_5$: MH, 511.2417. Found: 511.2428 ($MH^+$).

***N*-[4-(*N'*-(2,4-Diaminopteridin-6-ylmethyl))amino-3-methylbenzoyl]-L-glutamic Acid (3d)** To a suspension of **12d** (580 mg) in EtOH (40 ml) was added 1 N NaOH (3.5 ml) and the mixture was stirred at 25 °C overnight. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (10 ml). This solution was acidified to pH 3.7 with 1 N HCl and placed in a refrigerator for 2 h. The aqueous layer was centrifuged (2000 rpm, 15 min) and the obtained precipitate was lyophilized to give **3d** (331 mg, 65%) as an orange powder. $^1$H-NMR (DMSO-$d_6$) $\delta$: 1.8—2.4 (4H, m), 2.25 (3H, s), 4.40 (1H, m), 4.60 (2H, d, $J$=4.9 Hz), 6.18 (1H, m), 6.56 (1H, d, $J$=8.3 Hz), 7.56 (2H, m), 8.08 (1H, d, $J$=7.8 Hz), 8.71 (1H, s). IR (KBr) $cm^{-1}$: 3500—3000, 1640, 1610, 1510. FAB-MS *m/z*: 455 ($MH^+$). mp 204—207 °C (dec.). Analysis by HPLC (solvent, $CH_3COOH/CH_3COONa$, pH 5.4 : MeOH = 84 : 16; flow rate, 1.0 $cm^3$/min; detection, 254 nm) showed the purity to be at least 99% (retention time 17 min).

**Dimethyl *N*-(4-(*N'*-Benzyloxycarbonyl-*N'*-methylamino)-3-methylbenzoyl)-L-homoglutamate (5e)** Using the same procedure as described for the preparation of **5a**, **5e** was prepared from compound **4a** and dimethyl homoglutamate·hydrochloride. The yield of **5e** was 94%. Colorless oil. $^1$H-NMR ($CDCl_3$) $\delta$: 1.5—2.6 (9H, m), 3.15 (3H, s), 3.60 (3H, s), 3.72 (3H, s), 4.5—5.0 (1H, m), 5.05 (2H, s), 6.8—7.4 (7H, m), 7.5—8.0 (2H, m). IR (neat) $cm^{-1}$: 3350, 2960, 1740, 1660, 1610. MS *m/z*: 470 ($M^+$), 282, 91.

**Dimethyl *N*-(3-Methyl-4-methylaminobenzoyl)-L-homoglutamate (6e)** Using the same procedure as described for the preparation of **6a**, **6e** was prepared from compound **5e**. The yield of **6e** was 50%. Pale brown oil. $^1$H-NMR ($CDCl_3$) $\delta$: 1.4—2.0 (4H, m), 2.03 (3H, s), 2.34 (2H, t, $J$=6.0 Hz), 2.90 (3H, s), 3.63 (3H, s), 3.73 (3H, s), 4.5—5.0 (1H, m), 6.3—6.8 (2H, m), 7.3—7.7 (2H, m). IR (neat) $cm^{-1}$: 3600—3400, 2950, 1740, 1720, 1620. MS *m/z*: 336 ($M^+$), 148.

**Dimethyl *N*-[4-(*N'*-(2,4-Diaminopteridin-6-ylmethyl)-*N'*-methylamino)-3-methylbenzoyl]-L-homoglutamete (8e)** Using the same procedure as described for the preparation of **8a**, **8e** was prepared from **6e** and **7**. The yield of **8e** was 49%. Yellow powder. $^1$H-NMR ($CDCl_3$–$CD_3OD$) $\delta$: 1.5—2.0 (4H, m), 2.2—2.5 (5H, m), 2.74 (3H, s), 3.67 (3H, s), 3.79 (3H, s), 4.33 (2H, s), 4.7—4.9 (1H, m), 5.34 (2H, s), 6.78 (1H, d, $J$=6.8 Hz), 7.05 (1H, d, $J$=8.4 Hz), 7.61 (1H, d, $J$=7.8 Hz), 7.68 (1H, s), 8.85 (1H, s). IR (KBr) $cm^{-1}$: 3500—3300, 2950, 1740, 1630. MS *m/z*: 510 ($M^+$), 148. HR-FAB-MS *m/z*: Calcd for $C_{25}H_{31}N_8O_5$: MH, 511.2417. Found: 511.2407 ($MH^+$).

***N*-[4-(*N'*-(2,4-Diaminopteridin-6-ylmethyl)-*N'*-methylamino)-3-methylbenzoyl]-L-homoglutamic Acid (3e)** Using the same procedure as described for the preparation of **3a**, **3e** was prepared from **8e**. The yield of **3e** was 84%. Yellow powder. $^1$H-NMR (DMSO-$d_6$) $\delta$: 1.4—1.9 (4H, m), 2.24 (2H, t, $J$=7.8 Hz), 2.41 (3H, s), 2.72 (3H, s), 4.2—4.4 (3H, m), 7.11 (1H, d, $J$=8.8 Hz), 7.66 (1H, d, $J$=8.3 Hz), 7.74 (1H, s), 8.38 (1H, d, $J$=8.3 Hz), 8.64 (1H, s). IR (KBr) $cm^{-1}$: 3400—3200, 2960, 1620. FAB-MS *m/z*: 483 ($MH^+$). mp 211—213 °C (dec.). Analysis by HPLC (solvent, $CH_3COOH/CH_3COONa$, pH 5.4 : MeOH = 4 : 1; flow rate, 1.0 $cm^3$/min; detection, 254 nm) showed the purity to be at least 96% (retention time 31 min).

**1-Acetyl-7-methylindoline** A mixture of 7-methylindoline[9] (10.2 g) and acetic anhydride (25 ml) was refluxed for 30 min. The mixture was cooled to room temperature and poured into ice-water. The precipitated solid was collected by filtration and dried under vacuum to give 1-acetyl-7-methylindoline (11.7 g, 81%) as a white powder. $^1$H-NMR ($CDCl_3$) $\delta$: 2.24 (3H, s), 2.25 (3H, s), 2.99 (2H, t, $J$=7 Hz), 4.06 (2H, t, $J$=7 Hz), 7.01 (3H, m). IR (KBr) $cm^{-1}$: 1650, 1590, 1460, 1390, 1350. MS *m/z*: 175 ($M^+$), 134, 108, 91.

**1-Acetyl-5-bromo-7-methylindoline** To a solution of 1-acetyl-7-indoline (11.5 g) in AcOH (70 ml) was added bromine (3.1 ml) at room temperature and the mixture was stirred for 1 h, then poured into cold aqueous $Na_2S_2O_3$ solution. The precipitated solid was collected by filtration, and dried under vacuum to give 1-acetyl-5-bromo-7-methylindoline (10.8 g, 71%) as a white powder. $^1$H-NMR ($CDCl_3$) $\delta$:

2.24 (6H, s), 2.98 (2H, t, $J=7$ Hz), 4.06 (2H, t, $J=7$ Hz), 7.14 (2H, m). IR (KBr) $cm^{-1}$: 1660, 1460, 1440. MS *m/z*: 255 ($M^+ +2$), 253 ($M^+$), 211.

**1-Acetyl-5-cyano-7-methylindoline** A mixture of 1-acetyl-5-bromo-7-methylindoline (10.8 g) and CuCN (5.5 g) in 1-methylpyrrolidine (50 ml) was refluxed for 4 h, then cooled to room temperature, and poured into 30% $NH_4OH$. The precipitated solid was collected by filtration and dissolved in hot $CHCl_3$. This solution was filtered and the filtrate was washed successively with 30% $NH_4OH$, 1 N NaOH and water. The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was dried under vacuum to give 1-acetyl-5-cyano-7-methylindoline (4.9 g, 58%) as a white powder. $^1$H-NMR ($CDCl_3$) $\delta$: 2.24 (3H, s), 2.27 (3H, s), 3.05 (2H, t, $J=7$ Hz), 4.08 (2H, t, $J=7$ Hz), 7.30 (2H, m). IR (KBr) $cm^{-1}$: 2220, 1670, 1470. MS *m/z*: 200 ($M^+$), 158.

**7-Methylindoline-5-carboxylic Acid** A solution of 1-acetyl-5-cyano-7-methylindoline (4.9 g) in concentrated HCl (60 ml) was refluxed for 3 h, then cooled to room temperature, and poured into ice-water. The aqueous layer was adjusted to pH 12 with 1 N NaOH and filtered. The obtained filtrate was acidified to pH 4 with 3 N HCl and the precipitated white solid was collected by filtration, and dried under vacuum to give 7-methylindoline-5-carboxylic acid (3.4 g, 80%) as a white powder. $^1$H-NMR (DMSO-$d_6$) $\delta$: 2.05 (3H, s), 2.96 (2H, t, $J=8$ Hz), 3.54 (2H, t, $J=8$ Hz), 7.42 (2H, m), 11.83 (1H, br s). IR (KBr) $cm^{-1}$: 3340, 1670, 1610, 1430. MS *m/z*: 177 ($M^+$), 132.

**1-Benzyloxycarbonyl-7-methylindoline-5-carboxylic Acid (4f)** To a mixture of 7-methylindoline-5-carboxylic acid (3.3 g), 2 N NaOH (50 ml) and ether (60 ml) was added a solution of benzyloxycarbonyl chloride (6.8 ml) in ether (15 ml) at 0 °C and the mixture was stirred for 4 h at room temperature. The organic layer was separated, acidified to pH 2.0 with 3 N HCl and filtered. The obtained solid was washed with water, and dried under vacuum to give **4f** (5.2 g, 90%) as a white powder. $^1$H-NMR (DMSO-$d_6$) $\delta$: 2.22 (3H, s), 3.02 (2H, t, $J=7$ Hz), 4.10 (2H, t, $J=7$ Hz), 5.16 (2H, s), 7.32 (5H, m), 7.55 (2H, m). IR (KBr) $cm^{-1}$: 1730, 1670, 1440, 1410, 1390. MS *m/z*: 311 ($M^+$), 91. HR-MS *m/z*: Calcd for $C_{18}H_{17}NO_4$: M, 311.1157. Found: 311.1141 ($M^+$).

**Dimethyl *N*-(1-Benzyloxycarbonyl-7-methylindoline-5-carbonyl)-L-homoglutamate (5f)** Using the same procedure as described for the preparation of **5a**, **5f** was prepared from **4f** and dimethyl L-homoglutamate·hydrochloride. The yield of **5f** was 95%. Colorless oil. $^1$H-NMR ($CDCl_3$) $\delta$: 1.5—2.1 (4H, m), 2.2—2.6 (2H, m), 2.29 (3H, s), 3.00 (2H, t, $J=7$ Hz), 3.64 (3H, s), 3.76 (3H, s), 4.14 (2H, t, $J=7$ Hz), 4.5—5.0 (1H, m), 5.18 (2H, s), 6.67 (1H, d, $J=8$ Hz), 7.32 (5H, m), 7.44 (2H, m). IR (neat) $cm^{-1}$: 2960, 1720, 1640, 1610, 1600. MS *m/z*: 482 ($M^+$), 91. HR-MS *m/z*: Calcd for $C_{26}H_{30}N_2O_7$: M, 482.2053. Found: 482.2082 ($M^+$).

**Dimethyl *N*-(7-Methylindoline-5-carbonyl)-L-homoglutamate (6f)** Using the same procedure as described for the preparation of **6a**, **6f** was prepared from **5f**. The yield of **6f** was 89%. Colorless oil. $^1$H-NMR ($CDCl_3$) $\delta$: 1.5—2.1 (4H, m), 2.15 (3H, s), 2.38 (2H, t, $J=7$ Hz), 3.05 (2H, t, $J=7$ Hz), 3.60 (2H, t, $J=7$ Hz), 3.66 (3H, s), 3.73 (3H, s), 4.3—4.8 (1H, m), 7.46 (2H, m), 8.12 (1H, d, $J=7.3$ Hz). IR (KBr) $cm^{-1}$: 3500—3200, 2950, 1730, 1630. MS *m/z*: 348 ($M^+$), 160.

**Dimethyl *N*-[1-(2,4-Diamino-6-pteridinylmethyl)-7-methylindoline-5-carbonyl]-L-homoglutamate (8f)** Using the same procedure as described for the preparation of **8a**, **8f** was prepared from **6f** and **7**. The yield of **8f** was 66%. Yellow powder. $^1$H-NMR ($CDCl_3$–$CD_3OD$) $\delta$: 1.5—2.2 (4H, m), 2.3—2.7 (2H, m), 2.40 (3H, s), 2.8—3.9 (4H, m), 3.69 (3H, s), 3.78 (3H, s), 4.6—5.1 (1H, m), 4.77 (2H, s), 7.46 (3H, m), 8.75 (1H, s). IR (neat) $cm^{-1}$: 3400—3100, 2950, 1740, 1620, 1580. MS *m/z*: 522 ($M^+$), 350, 172, 160. HR-FAB-MS *m/z*: Calcd for $C_{25}H_{31}N_8O_5$: MH, 523.2417. Found: 523.2405 ($MH^+$).

***N*-[1-(2,4-Diamino-6-pteridinylmethyl)-7-methylindoline-5-carbonyl]-L-homoglutamic Acid (3f)** Using the same procedure as described for the preparation of **3a**, **3f** was prepared from **8f**. The yield of **3f** was 57%. Yellow powder. $^1$H-NMR (DMSO-$d_6$) $\delta$: 1.4—2.0 (4H, m), 2.23 (2H, t, $J=7$ Hz), 2.34 (3H, s), 2.97 (2H, t, $J=8$ Hz), 3.56 (2H, t, $J=8$ Hz), 4.32 (1H, m), 4.72 (2H, s), 7.44 (1H, s), 7.49 (1H, s), 8.14 (1H, d, $J=8$ Hz), 8.72 (1H, s). IR (neat) $cm^{-1}$: 3370, 3220, 2960, 1640, 1560. FAB-MS *m/z*: 481 ($MH^+$). mp 199—203 °C. Analysis by HPLC (solvent, $CH_3COOH/CH_3COONa$, pH 5.4 : MeOH = 4 : 1; flow rate, 1.0 $cm^3$/min; detection, 254 nm) showed the purity to be at least 96% (retention time 21 min).

**Dimethyl *N*-(3-Methyl-4-nitrobenzoyl)-L-homoglutamate (10g)** Using the same procedure as described for the preparation of **10d**, **10g** was prepared from 4-nitro-3-methylbenzoic acid and dimethyl L-homoglutamate·hydrochloride. The yield of **10g** was 94%. Colorless oil. $^1$H-NMR ($CDCl_3$) $\delta$: 1.6—2.1 (4H, m), 2.39 (2H, m), 2.62 (3H, s), 3.68 (3H, s), 3.80 (3H, s), 4.79 (2H, m), 7.13 (1H, m), 7.79 (2H, m), 7.98 (1H, d, $J=8.3$ Hz). IR (neat) $cm^{-1}$: 3400—3300, 2960, 1740, 1650, 1530. MS *m/z*: 352 ($M^+$), 321, 261, 89.

**Dimethyl *N*-(4-Amino-3-methylbenzoyl)-L-homoglutamate (11g)** Using the same procedure as described for the preparation of **11d**, **11g** was prepared from **10g**. The yield of **11g** was 52%. Colorless oil. $^1$H-NMR ($CDCl_3$) $\delta$: 1.6—2.1 (4H, m), 2.19 (3H, s), 2.36 (2H, m), 3.66 (3H, s), 3.77 (3H, s), 4.79 (1H, m), 6.63 (2H, m), 7.55 (2H, m). IR (neat) $cm^{-1}$: 3400—3300, 2950, 1740, 1640, 1600. MS *m/z*: 322 ($M^+$), 222, 150, 134.

**Dimethyl *N*-[4-(*N'*-(2,4-Diaminopteridin-6-ylmethyl))amino-3-methylbenzoyl]-L-homoglutamate (12g)** Using the same procedure as described for the preparation of **12d**, **12g** was prepared from **11g**. The yield of **12g** was 35%. Brown powder. $^1$H-NMR ($CDCl_3$–$CD_3OD$) $\delta$: 1.6—2.0 (4H, m), 2.29 (3H, s), 2.39 (2H, m), 3.68 (3H, s), 3.77 (3H, s), 4.65 (2H, s), 4.74 (1H, m), 6.62 (1H, d, $J=9.3$ Hz), 7.18 (1H, d, $J=7.3$ Hz), 7.60 (2H, m), 8.77 (1H, s). IR (KBr) $cm^{-1}$: 3500—3300, 2960, 1740, 1630, 1510. MS *m/z*: 496 ($M^+$), 436, 308, 175, 150, 134. HR-FAB-MS *m/z*: Calcd for $C_{23}H_{29}N_8O_5$: MH, 497.2261. Found: 497.2290 ($MH^+$).

***N*-[4-(*N'*-(2,4-Diaminopteridin-6-ylmethyl))amino-3-methylbenzoyl]-L-homoglutamic Acid (3g)** Using the same procedure as described for the preparation of **3d**, **3g** was prepared from **12g**. The yield of **3g** was 54%. Orange powder. $^1$H-NMR (DMSO-$d_6$) $\delta$: 1.5—1.9 (4H, m), 2.24 (5H, m), 4.34 (1H, m), 4.58 (2H, m), 6.21 (1H, m), 6.56 (1H, d, $J=8.8$ Hz), 7.59 (2H, m), 8.05 (1H, d, $J=7.3$ Hz), 8.70 (1H, s). IR (KBr) $cm^{-1}$: 3500—3200, 1640, 1610, 1510. FAB-MS *m/z*: 469 ($MH^+$). mp 208—212 °C (dec.). Analysis by HPLC (solvent, $CH_3COOH/CH_3COO$-Na, pH 5.4 : MeOH = 4 : 1; flow rate, 1.0 $cm^3$/min; detection, 254 nm) showed the purity to be at least 99% (retention time 16 min).

**Peripheral Blood Mononuclear Cell Culture** PBMC from a healthy donor were separated by centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden). Cells were resuspended in RPMI 1640 medium containing 5% fetal bovine serum (FBS: Hyclone Laboratories Inc., Logan, UT), glutamine, penicillin G and streptomycin. Cells ($1 \times 10^5$ cells/well) were cultured in 0.2 ml wells in 96-well microtiter plates (Corning #25870) with phytohemagglutinin (0.3 $\mu$g/ml) (PHA: Welcome Foundation Ltd., Dartford, UK) for 3 d. [$^3$H]Deoxyuridine (UdR: 1 $\mu$Ci/well) (Amersham International plc, Buckinghamshire, UK) was added to each well for the last 5 h of culture and the proliferation was assessed by determining [$^3$H]UdR uptake into the cells.

**Synovial Fibroblastic Cell Culture** Synovial tissues were obtained from RA patients at the time of joint surgery. The tissue was minced and enzymatically dissociated with 5 mg/ml of collagenase (type I: Sigma Chemical Co.) and 0.15 mg/ml of DNase (from bovine pancreas: Sigma Chemical Co.) in Iscove's modified Dulbecco's medium (IMDM: Gibco) for 1 h at 37 °C. The resulting cells were plated in culture flask and allowed to adhere, and the nonadherent cells were removed. SC were used for proliferation assay in the third to sixth passage. SC were resuspended in IMDM medium containing 5% FBS supplemented with penicillin G and streptomycin. Cells ($3 \times 10^3$ cells/well) were cultured in 0.2 ml wells in 96-well microtiter plates (Falcon #3072) for 5 d. [$^3$H]UdR (1 $\mu$Ci/well) was added for the last 2 d of culture and the proliferation was assessed by determining [$^3$H]UdR uptake.

**Induction of Adjuvant Arthritis** Induction of adjvant arthritis was done as previously reported.[6] Briefly, male rats (Lewis, 6-week-old) were inoculated into the base of the tail with 50 $\mu$l of liquid paraffin containing 35 $\mu$g of heat-killed *Mycobacterium tuberculosis* H37 Ra (Difco Laboratories, Detroit, MI) . The system described by Trentham *et al.*[7] was used to assess the severity of the arthritis. Each paw was graded from 0 to 4 based on erythema, swelling, and deformity of the joints.

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**References**

1) Matsuoka H., Kato N., Tsuji K., Maruyama N., Suzuki H., Mihara M., Takeda Y., Yano K., *Chem. Pharm. Bull.*, **44**, 1332—1337 (1996).
2) Galivan J., *Mol. Pharmacol.*, **17**, 105—110 (1980).
3) Chabner B. A., Allegra C. J., Curt G. A., Clendeninn N. J., Baram J., Koizumi S., *J. Clin. Invest.*, **76**, 907—912 (1985).

4) Piper J. R., Montgomery J. A., *J. Org. Chem.*, **42**, 208—211 (1977).
5) Moran R. G., Colman P. D., Rosowsky A., Forsch R. A., Chan K. K., *Mol. Pharmacol.*, **27**, 156—166 (1984).
6) Mihara M., Ikuta M., Koishihara Y., Ohsugi Y., *Eur. J. Immunol.*, **21**, 2327—2331 (1991).
7) Trentham D. E., Townes A. S., Kang A. H., *J. Exp. Med.*, **146**, 857—868 (1977).
8) Lofgrin N., Tegner C., Willman N.E., Dahlbom R., *Acta Pharm. Suecica.*, **2**, 79—86 (1965).
9) Gribble G. W., Hofmann J. H., *Synthesis*, **12**, 859—860 (1977).