Antirheumatic Agents: Novel Methotrexate Derivatives Bearing a Benzoxazine or Benzothiazine Moiety

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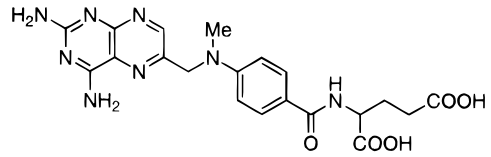
Novel methotrexate (MTX) derivatives bearing dihydro-2*H*-1,4-benzothiazine or dihydro-2*H*-1,4-benzoxazine were synthesized and tested for *in vitro* antiproliferative activities against human synovial cells (hSC) and human peripheral blood mononuclear cells (hPBMC) obtained from patients with rheumatoid arthritis and healthy volunteers, respectively. *In vivo* antiarthritic activities of these derivatives were also evaluated in a rat adjuvant arthritis model. *N*-[[4-[(2,4-Diaminopteridin-6-yl)methyl]-3,4-dihydro-2*H*-1,4-benzothiazin-7-yl]carbonyl]-L-glutamic acid (**3c**) exhibited more potent antiproliferative activities in hSC and hPBMC than MTX *in vitro*. Antiproliferative activities of *N*-[[4-[(2,4-diaminopteridin-6-yl)methyl]-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl]carbonyl]-L-homoglutamic acid (**3b**) and *N*-[[4-[(2,4-diaminopteridin-6-yl)methyl]-3,4-dihydro-2*H*-1,4-benzothiazin-7-yl]carbonyl]-L-homoglutamic acid (**3d**) (MX-68) were comparable to that of MTX in these *in vitro* assays. Compounds **3b**,**d** (MX-68) significantly suppressed progression of the adjuvant arthritis in a dose-dependent manner ranging from 0.5 to 2.5 mg/kg (po). In addition, **3d** (MX-68) completely suppressed this progression at the dose of 2.5 mg/kg (po). Importantly, **3d** (MX-68) having benzothiazine and homoglutamate, as expected, did not undergo polyglutamation, a process which may be responsible for the associated side effects of MTX. These results suggest that **3d** (MX-68) is a potent and safe candidate antirheumatic agent, absent of the side effects of MTX.

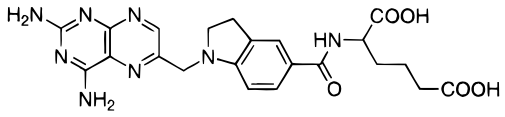
Introduction

Methotrexate (MTX; Figure 1) was synthesized about 50 years ago and is still used as an antileukemic agent because of its high anti-folate activity. Based on its biological profile, it is effective for the treatment of rheumatoid arthritis (RA),[1] psoriasis,[2] and other autoimmune diseases[3,4] and has been improved over recent years.[5,6] However, long term MTX therapy is associated with some serious side effects, such as hepatic dysfunction[7] and lung fibrosis.[8]

MTX *via* its metabolites is thought to exert both its biological action and side effects because it acts as a substrate for folylpolyglutamate synthetase (FPGS), which catalyzes the formation of intracellular polyglutamates at the γCOOH group extending from glutamate within MTX.[9] This potentially leads to inclusion bodies that elicit cellular disruption and subsequent inflammation.[10] Thus we initially attempted to suppress this FPGS substrate activity and enhance dihydrofolate reductase (DHFR) binding by designing a compound which is not metabolically altered. This was done by synthesizing the MTX derivative MX-33 (**2**), which has a homoglutamate and an indoline ring in place of the glutamate and aminobenzoic acid, respectively (Figure 1).[11] MX-33 did not act as a substrate for FPGS because polyglutamation could not occur in the absence of the γCOOH. The use of the indoline was based on our interpretation of Oefner's model which examined binding between MTX and DHFR.[12] According to this model, we hypothesized the existence of a hydrophobic open space between the DHFR and the aminobenzoic acid moiety of MTX. We



Methotrexate (MTX) **1**



MX-33 **2**

Figure 1.

speculated that filling this space with a fixed hydrophobic substituent would cause tighter and energetically more favorable binding to DHFR and thus enhance the anti-DHFR activity.

MX-33 potently inhibited proliferation of human synovial cells (hSC) and human peripheral blood mononuclear cells (hPBMC) obtained from patients with RA and healthy volunteers, respectively. It also significantly suppressed progression of adjuvant arthritis at a dose of 5.0 mg/kg (po).

To further enhance DHFR binding, considerable attention was paid to the indoline moiety. After considering both Oefner's model and our binding studies,[13] we predicted that we could potentiate binding to DHFR without changing the direction and orientation of the pteridine ring if we replaced the indoline ring of MX-33 with the slightly enlarged dihydro-2*H*-1,4-benzoxazine or dihydro-2*H*-1,4-benzothiazine. In our model, the carbon atom adjacent to the phenyl ring in this indoline moiety was partially exposed to the solvent

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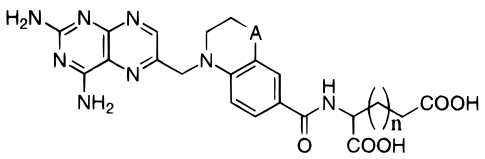
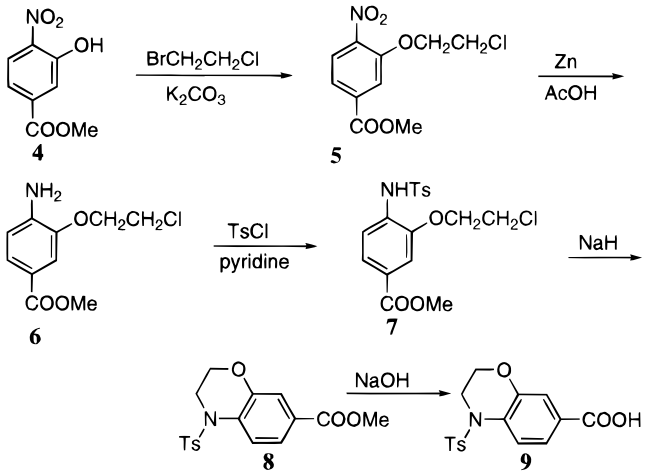


	A	n
3a	O	1
3b	O	2
3c	S	1
3d	S	2 (MX-68)
3e	SO	1

Figure 2.

Scheme 1



portion; therefore, insertion of either an oxygen or sulfur atom between the methylene group and benzene ring was expected to energetically favor its location toward the solvent portion and gain a more stable interaction with solvent water molecules rather than the carbon atom. Insertion of a sulfur atom was expected to enhance DHFR binding by van der Waals interactions.

In this paper, we report the design, synthesis, and biological properties of novel MTX derivatives bearing a dihydro-2*H*-1,4-benzoxazine or dihydro-2*H*-1,4-benzothiazine moiety (Figure 2).

Chemistry

Preparations of the key intermediates of *N*-(4′-tolylsulfonyl)-3,4-dihydro-2*H*-1,4-benzoxazine-7-carboxylic acid (**9**) and *N*-(4′-tolylsulfonyl)-3,4-dihydro-2*H*-1,4-benzothiazine-7-carboxylic acid (**17**) are presented in Schemes 1 and 2, respectively. Synthesis of the target compounds **3a**−**d** is presented in Scheme 3.

As shown in Scheme 1, synthesis of intermediate **9** started with *O*-alkylation of the phenol **4** with 1-bromo-2-chloroethane to give ether **5**, and its following reduction with zinc powder in acetic acid yielded the corresponding amine **6**. The amino group of **6** was then protected with a 4-toluenesulfonyl group to give sulfonyl amide **7**. Subsequent cyclization of **7** proceeded effectively by the treatment of NaH to yield benzoxazine ester **8**, and hydrolysis of **8** with 1 N NaOH produced the key intermediate **9**.

As shown in Scheme 2, synthesis of intermediate **17** was achieved using a slight modification of Wolfe's method.[14] Initially, starting material **10** was converted into benzothiazol **11** by treatment with sodium thiocyanate in the presence of bromine. Alkali hydrolysis of **11** gave thiol **12**, which was immediately submitted to *S*-alkylation with 1-bromo-2-chloroethane in aqueous solvent to give thioether **13**. Esterification of **13** was achieved by the treatment of HCl−MeOH to give **14**, which was protected with a 4-toluenesulfonyl group to give sulfonyl amide **15**. Subsequently, **15** was cyclized by the treatment of NaH to give benzothiazine ester **16**. Compound **16** was then hydrolyzed with 1 N NaOH to produce the key intermediate **17**.

As shown in Scheme 3, the synthesis of the target compounds **3a**−**d** was achieved using one standard synthetic method.[15] The two key intermediates, **9** and **17**, were converted to the corresponding acid chlorides. Subsequently, their coupling with amino diesters was performed using the Schotten Baumann's procedure to give amides **18a**−**d**. The toluenesulfonyl group of **18a**−**d** was then removed by HBr in acetic acid to give deprotected amines **19a**−**d**, which were effectively alkylated with 6-(bromomethyl)-2,4-diaminopteridine[16] (**20**) to produce **21a**−**d**. Finally, **21a**−**d** were hydrolyzed with 1 N NaOH to yield the target compounds **3a**−**d**.

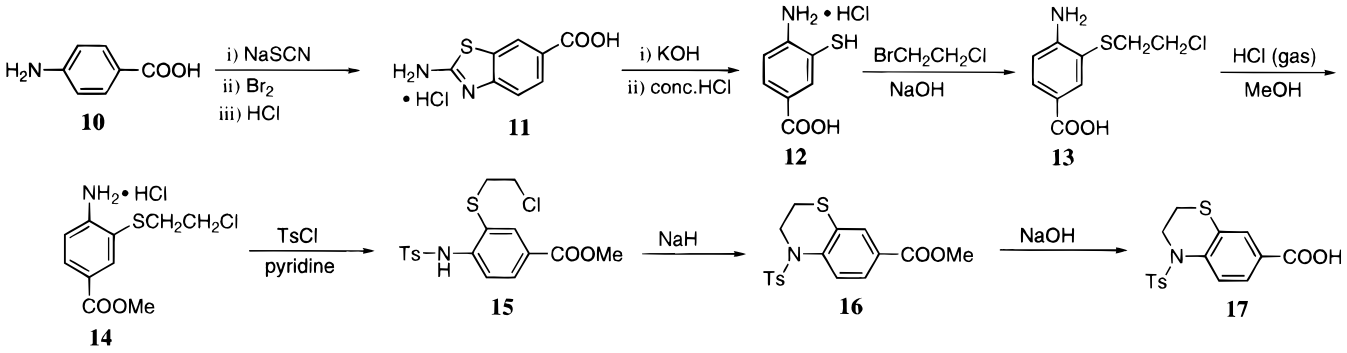
Results and Discussions

The novel MTX derivatives synthesized were tested for antiproliferative activity in hSC and hPBMC *in vitro*. As shown in Table 1, all tested compounds suppressed proliferation in hSC with differing potency; glutamate derivatives **3a,c** were more potent than MTX with IC_{50} values of 32 and 18 nM, respectively, whereas that of MTX was 61 nM. Antiproliferative effects of homoglutamate derivatives **3b**,**d** (MX-68) were comparable to that of MTX with IC_{50} values of 87 and 47 nM and were 3 and 5 times more potent than that of the homoglutamate derivative MX-33, respectively. These results may support our hypothesis that DHFR binding affinity is enhanced after enlargement and insertion of the heteroatom within the indoline ring. All tested compounds potently suppressed proliferation of hPBMC; compounds more potent than MTX were **3c** and MX-33 with IC_{50} values of 12 and 20 nM, respectively. The calculated relative value in hSC for **3d** (MX-68) was considerably less than that for MX-33, whereas it was 1.5 times greater than that for MX-33 in hPBMC. Thus **3d** (MX-68) is more selective for hSC than for hPBMC. In light of the role played by SC during synovial inflammation, MX-68's potent action toward these cells supports its use as an antirheumatic agent.

In both hSC and hPBMC assays, the inhibitory effects of the glutamate derivatives **3a**,**c** were 2−3 times enhanced over their corresponding homoglutamate counterparts **3b**,**d** (MX-68). These results indicate that this enhanced action occurs *via* polyglutamation. We corroborated this concept by using Moran's method[17] to show the presence and absence of substrate activity for FPGS by MTX and **3d** (MX-68), respectively (Table 2).

Antirheumatic activities of our novel MTX derivatives were assessed *in vivo* by their effects on rat adjuvant arthritis.[18] Table 3 compares the effects of these derivatives to MTX; the suppressive effect of **3a** was comparable at the dose of 0.25 mg/kg (po), whereas the

Scheme 2



Scheme 3

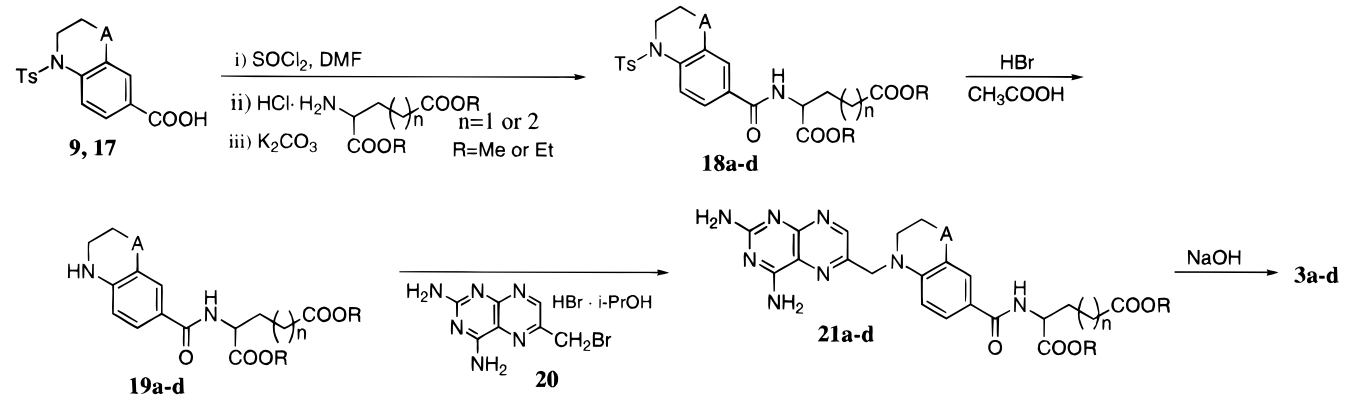


Table 1. Antiproliferative Activities of Novel MTX Derivatives

compd	IC_{50} (nM)[a,b] hSC	hPBMC
3a	32 (0.52)	24 (1.0)
3b	87 (1.4)	45 (1.9)
3c	18 (0.30)	12 (0.50)
3d (MX-68)	47 (0.77)	32 (1.3)
MX-33	230 (3.8)	20 (0.83)
MTX	61 (1.0)	24 (1.0)

[a] The results are the mean of triplicate assays. [b] Numbers in parentheses are normalized relative to the IC_{50} for each compound against the IC_{50} of MTX.

Table 2. Activities of MTX Derivatives as Substrates of FPGS[a]

compd	drug concentration (μM)	FPGS activities (nmol/mg/h)
MTX	100	2.64
MX-68	100	<0.01
MX-68	500	0.07

[a] FPGS activity was determined as described in ref 17, with partially purified enzyme from rat liver. The results are the mean of triplicate assays.

effect of **3c** was obviously more potent. Furthermore, homoglutamate derivatives **3b**,**d** (MX-68) suppressed progression of the arthritis in a dose-dependent manner from 0.5 to 2.5 mg/kg (po). More importantly, **3d** (MX-68) completely suppressed the arthritis at the dose of 2.5 mg/kg. It was more effective than MX-33 at the same dose and comparable to MTX at 0.25 mg/kg. Furthermore, greater *in vitro* potency of **3d** (MX-68) over MX-33 in hSC is consistent with the effects observed *in vivo*.

As polyglutamation causes side effects but is prominently implicated for the efficacy of MTX, derivatives which do not have a capability to undergo this process must compensate by some other means to achieve sufficient *in vivo* pharmacological effects. In this respect, the homoglutamate derivative **3d** (MX-68), found to be dose-dependent and orally active in the rat adjuvant arthritis and not to undergo polyglutamation, is a strong candidate antirheumatic agent. MX-68 is currently under preclinical investigation with the aim of clinical study in the near future.

Table 3. Effects of Novel MTX Derivatives on the Development of Adjuvant Arthritis in Rats

compd	dose (mg/kg)	% suppression of arthritis	
3a	0.05	48	($p < 0.02$)
	0.25	94	($p < 0.02$)
3b	0.25	18	($p < 0.05$)
	0.5	32	($p < 0.05$)
	1.0	53	($p < 0.02$)
	2.5	84	($p < 0.02$)
3c	0.05	52	(NS)
	0.1	97	($p < 0.02$)
3d (MX-68)	0.25	20	(NS)
	0.5	58	($p < 0.02$)
	1.0	74	($p < 0.02$)
	2.5	94	($p < 0.02$)
MX-33	2.5	26	(NS)
	5.0	38	(NS)
MTX	0.1	75	($p < 0.02$)
	0.25	92	($p < 0.02$)

Experimental Section

^{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 Model 270-3 infrared spectrometer, and EI mass spectra were recorded on a Shimadzu GCMS-QP1000 instrument. FAB and HR-FAB mass spectra were recorded on a VG Analytical VG11-250 instrument. TLC was routinely performed on Merck Kieselgel 60 F254. HPLC analysis were performed on a Hitachi L-3000 (detector) with Hitachi L-6200 (pump); the column was YMC-Pack A-312 S-5 120A ODS. Melting points were taken on a Yanaco Model MP apparatus.

Methyl 3-(2′-Bromoethoxy)-4-nitrobenzoate (5). To a solution of methyl 3-hydroxy-4-nitrobenzoate (**4**) (1.0 g, 5.07 mmol) in dimethylformamide (DMF; 10 mL) were added 1,2-dibromoethane (0.5 mL, 5.80 mmol) and potassium carbonate (700 mg, 5.07 mmol) at room temperature, and the mixture was stirred for 2 h at 100 °C. The mixture was then poured into water and extracted with toluene. The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The obtained residue was chromatographed on silica gel with *n*-Hex−AcOEt (3:1) to give **5** (860 mg, 56%) as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 3.69 (t, 2H, J = 6 Hz, -OCH_2-), 3.95 (s, 3H, -OCH_3), 4.49 (t, 2H, J = 6 Hz, -CH_2Br), 7.75 (m, 3H, Ar). IR (KBr): cm^{-1} 1720, 1610, 1590. MS: m/z 306 (M^+ + 2), 304 (M^+), 106.

Methyl 4-Amino-3-(2′-bromoethoxy)benzoate (6). To a solution of **5** (100 mg, 0.33 mmol) in acetic acid (2.0 mL) was added zinc powder (213 mg, 3.26 mmol) at 0 °C, and the reaction mixture was stirred for 2 h at room temperature and filtered. The filtrate was concentrated under reduced pressure to afford a brown crude oil, which was dissolved with $CHCl_3$. The organic solution was washed with 5% $NaHCO_3$, dried over Na_2SO_4, filtered, and concentrated to give pure **6** (75 mg, 83%) as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 3.68 (t, 2H, J = 5.7 Hz, -OCH_2-), 3.86 (s, 3H, OCH_3), 4.37 (t, 2H, J = 5.7 Hz, -CH_2Br), 6.68 (d, 1H, J = 8.3 Hz, Ar), 7.44 (m, 1H, Ar), 7.56 (d, 1H, J = 8.3 Hz, Ar). IR (KBr): cm^{-1} 3380, 1710, 1620. MS: m/z 276 (M^+ + 2), 274 (M^+), 166.

Methyl *N*-(4′-Tolylsulfonyl)-4-amino-3-(2′-bromoethoxy)-benzoate (7). A mixture of **6** (1.2 g, 4.38 mmol) and 4-toluenesulfonyl chloride (1.7 g, 8.90 mmol) in pyridine (10 mL) was stirred at room temperature overnight, and the mixture was poured into aqueous NH_4Cl solution. Precipitated white solids were collected by filtration, and the obtained solids were extracted with $CHCl_3$. The $CHCl_3$ layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residual oil was recrystallized from *n*-Hex−AcOEt to give **7** (750 mg, 40%) as a white solid. ^{1}H-NMR ($CDCl_3$): δ 2.36 (s, 3H, toluene-CH_3), 3.5−3.9 (m, 2H, -OCH_2-), 3.87 (s, 3H, -OCH_3), 4.24 (m, 2H, -CH_2Br), 7.22 (d, 2H, J = 8.3 Hz, toluene-Ar), 7.33 (m, 2H, benzoate-Ar + NH), 7.42 (s, 1H, benzoate-Ar), 7.62 (s, 1H, benzoate-Ar), 7.72 (d, 2H, J = 8.3 Hz, toluene-Ar). IR (KBr): cm^{-1} 3240, 1730, 1610. MS: m/z 429 (M^+ + 2), 427 (M^+), 91.

Methyl *N*-(4′-Tolylsulfonyl)-3,4-dihydro-2*H*-1,4-benzoxazine-7-carboxylate (8). To a solution of **7** (750 mg, 1.75 mmol) in DMF (23 mL) was added NaH (335 mg, 8.38 mmol, 60% in mineral oil) at 0 °C under nitrogen atmosphere, and the mixture was stirred for 1 h at the same temperature. The mixture was poured into aqueous NH_4Cl solution and extracted with toluene. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The obtained residue was chromatographed on silica gel with *n*-Hex−AcOEt (2:1) to give **8** (430 mg, 71%) as a white powder. ^{1}H-NMR ($CDCl_3$): δ 2.38 (s, 3H, toluene-CH_3), 3.80 (m, 2H, -NCH_2-), 3.88 (s, 3H, -OCH_3), 3.89 (m, 2H, -CH_2O-), 7.24 (d, 2H, J = 8.3 Hz, toluene-Ar), 7.4−7.6 (m, 4H, toluene-Ar2H + benzoxazine-Ar2H), 7.93 (d, 1H, J = 8.8 Hz, benzoxazine-Ar). IR (KBr): cm^{-1} 3600, 2950, 1720, 1620, 1600. MS: m/z 347 (M^+), 192.

***N*-(4′-Tolylsulfonyl)-3,4-dihydro-2*H*-1,4-benzoxazine-7-carboxylic Acid (9).** A mixture of **8** (350 mg, 1.01 mmol) and 1 N NaOH (2.9 mL) in ethanol (15 mL) was stirred for 5 h at room temperature, and the mixture was concentrated under reduced pressure. The obtained residue was diluted to 5 mL with water, and this solution was acidified to pH 2.0 with 1 N HCl. The precipitated solids were collected by filtration and dried *in vacuo* to give **9** (298 mg, 89%) as a white powder. ^{1}H-NMR ($CDCl_3$): δ 2.38 (s, 3H, toluene-CH_3), 3.83 (m, 2H, -NCH_2-), 3.92 (m, 2H, -CH_2O-), 7.24 (d, 2H, J = 8.3 Hz, toluene-Ar), 7.5−7.7 (m, 4H, toluene-Ar2H + benzoxazine-Ar2H), 7.93 (d, 1H, J = 8.8 Hz, benzoxazine-Ar). IR (KBr): cm^{-1} 3500−3400, 3100−2900, 1680. MS: m/z 333 (M^+), 178. Anal. ($C_{16}H_{15}NO_5S$) C, H, N, S.

Dimethyl *N*-[[4-(4′-Tolylsulfonyl)-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl]carbonyl]-L-homoglutamate (18b). A mixture of **9** (2.9 g, 8.71 mmol) and DMF (0.1 mL) in thionyl chloride (10 mL) was stirred for 2 h at room temperature and concentrated. The obtained residue was dissolved with CH_2Cl_2 (40 mL). To this solution were added a solution of dimethyl L-homoglutamate hydrochloride (2.8 g, 12.4 mmol) in water (40 mL) and potassium carbonate (5.0 g, 36.2 mmol). The whole mixture was vigorously stirred overnight, poured into water, and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The obtained residue was chromatographed on silica gel with $CHCl_3$−MeOH (100:1) to give **18b** (4.3 g, 98%) as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 1.6−2.1 (m, 4H, hGlu-β,γ), 2.35 (m, 2H, hGlu-δ), 2.39 (s, 3H, toluene-CH_3), 3.66 (s, 3H, hGluα-OCH_3), 3.77 (s, 3H, hGluβ-OCH_3), 3.72 (m, 2H, -NCH_2-), 3.87 (m, 2H, -CH_2O-), 4.74 (m, 1H, hGlu-α), 6.73 (d, 1H, J = 7.3 Hz, NH), 7.2−7.4 (m, 4H, toluene-Ar2H + benzoxazine-Ar2H), 7.51 (d, 2H, J = 8.3 Hz, toluene-Ar), 7.93 (d, 1H, J = 8.8 Hz, benzoxazine-Ar). IR (neat): cm^{-1} 3400, 2950, 1740, 1650. MS: m/z 504 (M^+), 91.

Dimethyl *N*-[(3,4-Dihydro-2*H*-1,4-benzoxazin-7-yl)carbonyl]-L-homoglutamate (19b). A mixture of **18b** (4.3 g, 8.53 mmol) and anisole (4.3 g, 39.8 mmol) in 30% HBr in acetic acid (50 mL) was stirred for 4 h at room temperature and poured into ether (300 mL). Precipitates were collected and washed by decantation with ether. The obtained residue was dissolved with $CHCl_3$, and this solution was washed with 5% $NaHCO_3$, dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give **19b** (1.45 g, 49%) as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 1.6−2.1 (m, 4H, hGlu-β,γ), 2.38 (t, 2H, J = 6.8 Hz, hGlu-δ), 3.46 (m, 2H, -NCH_2-), 3.68 (s, 3H, hGluα-OCH_3), 3.77 (s, 3H, hGluδ-OCH_3), 4.24 (m, 2H, -CH_2O-), 4.72 (m, 1H, hGlu-α), 6.59 (d, 1H, J = 8.3 Hz, Ar), 7.33 (m, 3H, NH + Ar2H). IR (neat): cm^{-1} 3600−3400, 2950, 1730, 1640, 1610. MS: m/z 350 (M^+), 162. HR-MS calcd for $C_{17}H_{22}N_2O_6$: M, 350.1478. Found: 350.1488 (M^+).

Dimethyl *N*-[[4-[(2,4-Diaminopteridin-6-yl)methyl]-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl]carbonyl]-L-homoglutamate (21b). A mixture of **19b** (1.45 g, 4.14 mmol) and 6-(bromomethyl)-2,4-diaminopteridine·HBr·*i*-PrOH[14] (**20**) (1.47 g, 3.71 mmol) in dimethylacetamide (DMA; 23 mL) was heated at 60 °C for 4 h with stirring. The mixture was poured into 5% $NaHCO_3$ and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The obtained residual oil was chromatographed on silica gel with $CHCl_3$−MeOH (10:1) to give **21b** (1.48 g, 68%) as a yellow powder. ^{1}H-NMR ($CDCl_3$−CD_3OD): δ 1.6−2.1 (m, 4H, hGlu-β,γ), 2.36 (t, 2H, J = 6.8 Hz, hGlu-α), 3.58 (m, 2H, -NCH_2-), 3.66 (s, 3H, hGluα-OCH_3), 3.76 (s, 3H, hGluδ-OCH_3), 4.39 (m, 2H, -CH_2O-), 4.67 (s, 2H, pteridine-CH_2), 4.73 (m, 1H, hGlu-α), 6.66 (d, 1H, J = 8.3 Hz, benzoxazine-Ar), 6.99 (d, 1H, J = 7.3 Hz, NH), 7.29 (m, 2H, benzoxazine-Ar), 8.70 (s, 1H, pteridine-Ar). IR (KBr): cm^{-1} 3500−3300, 3200, 2950, 1750, 1630, 1580. FAB-MS: m/z 525 (MH^+). HR-FAB-MS calcd for $C_{24}H_{28}N_8O_6$: MH^+, 525.2210. Found: 525.2200 (MH^+).

***N*-[[4-[(2,4-Diaminopteridin-6-yl)methyl]-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl]carbonyl]-L-homoglutamic Acid (3b).** A mixture of **21b** (1.48 g, 2.82 mmol) and 1 N NaOH (8.5 mL) in ethanol (80 mL) was stirred overnight at room temperature and concentrated to 5 mL under reduced pressure. The residue was diluted to 50 mL with water, and this aqueous solution was adjusted to pH 4 by 1 N HCl and 3% $(NH_4)_2CO_3$. Precipitates were collected by filtration, and obtained solids were dried *in vacuo* to give **3b** (1.26 g, 90%) as a yellow powder. ^{1}H-NMR (DMSO-d_6): δ 1.5−2.0 (m, 4H, hGlu-β,γ), 2.14 (t, 2H, J = 6.8 Hz, hGlu-δ), 3.68 (m, 2H, -NCH_2-), 4.28 (m, 3H, -CH_2O- + hGlu-α), 4.71 (s, 2H, pteridine-CH_2), 6.80 (d, 1H, J = 8.3 Hz, benzoxazine-Ar), 7.31 (m, 2H, benzoxazine-Ar), 8.13 (d, 1H, J = 7.3 Hz, NH), 8.71 (s, 1H, pteridine-Ar). IR (KBr): cm^{-1} 3500−3300, 1640, 1620. FAB-MS: m/z 497 (MH^+). HR-FAB-MS calcd for $C_{22}H_{25}N_8O_6$: MH^+, 497.1897. Found: 497.1980 (MH^+). Mp: 200−202 °C dec. Anal. ($C_{22}H_{24}N_8O_6$·$2H_2O$) C, H, N. Analysis of HPLC (solvent, CH_3CO_2H/CH_3CO_2Na (pH 5.4):MeCN = 9:1; flow rate, 1.0 cm^3/min; detection, 254 nm) showed the purity to be at least 98% (retention time, 18 min).

Diethyl *N*-[[4-(4′-Tolylsulfonyl)-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl]carbonyl]-L-glutamate (18a). Using the same procedure as described for the preparation of **18b**, compound

18a was prepared from compound **9** and diethyl L-glutamate hydrochloride. The yield of **18a** was 95% (colorless oil). ^{1}H-NMR ($CDCl_3$): δ 1.24 (t, 3H, J = 6.8 Hz, Gluα-OCH$_2$*CH$_3$*), 1.30 (t, 3H, J = 6.8 Hz, Gluγ-OCH$_2$*CH$_3$*), 2.40 (s, 3H, toluene-CH$_3$), 2.0−2.6 (m, 4H, Glu-β,γ), 3.75 (m, 2H, -NCH$_2$-), 3.90 (m, 2H, -CH$_2$O-), 4.13 (q, 2H, J = 6.8 Hz, Gluα-O*CH$_2$*CH$_3$), 4.24 (q, 2H, J = 6.8 Hz, Gluγ-O*CH$_2$*CH$_3$), 4.74 (m, 1H, Glu-α), 7.00 (d, 1H, J = 7.3 Hz, NH), 7.2−7.4 (m, 4H, toluene-Ar2H + benzoxazine-Ar2H), 7.52 (d, 2H, J = 8.3 Hz, toluene-Ar), 7.95 (d, 1H, J = 8.8 Hz, benzoxazine-Ar). IR (neat): cm^{-1} 2980, 1740, 1660, 1572. MS: m/z 518(M$^+$), 133. HR-MS m/z calcd for $C_{25}H_{30}N_2O_8S$: M, 518.1723. Found: 518.1722 (M$^+$).

Diethyl *N*-[(3,4-Dihydro-2*H*-1,4-benzoxazin-7-yl)carbonyl]-L-glutamate (19a). Using the same procedure as described for the preparation of **19b**, compound **19a** was prepared from compound **18a**. The yield of **19a** was 56% (white powder). ^{1}H-NMR ($CDCl_3$): δ 1.2−1.4 (m, 6H, Gluα, γ-OCH$_2$*CH$_3$*), 2.0−2.4 (m, 2H, Glu-β), 2.4−2.6 (m, 2H, Glu-γ), 3.41 (m, 2H, -NCH$_2$-), 4.0−4.3 (m, 6H, Gluα, γ-O*CH$_2$*CH$_3$ + -CH$_2$O-), 4.74 (m, 1H, Glu-α), 6.53 (d, 1H, J = 8.8 Hz, Ar), 6.94 (d, 1H, J = 7.8 Hz, NH), 7.22 (m, 2H, Ar). IR (neat): cm^{-1} 3500−3300, 1720, 1640, 1580. MS: m/z 364 (M$^+$), 162. HR-MS calcd for $C_{18}H_{24}N_2O_6$: M, 364.1634. Found: 364.1634 (M$^+$).

Diethyl *N*-[[4-[(2,4-Diaminopteridin-6-yl)methyl]-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl]carbonyl]-L-glutamate (21a). Using the same procedure as described for the preparation of **21b**, compound **21a** was prepared from compound **19a**. The yield of **21a** was 54% (orange powder). ^{1}H-NMR ($CDCl_3$−CD_3OD): δ 1.23 (t, 3H, J = 6.8 Hz, Gluα-OCH$_2$*CH$_3$*), 1.29 (t, 3H, J = 6.8 Hz, Gluγ-OCH$_2$*CH$_3$*), 2.0−2.3 (m, 2H, Glu-β), 2.3−2.5 (m, 2H, Glu-γ), 3.60 (m, 2H, -NCH$_2$-), 4.11 (m, 2H, Gluα-O*CH$_2$*CH$_3$), 4.22 (m, 2H, Gluγ-O*CH$_2$*CH$_3$), 4.32 (m, 2H, -CH2O-), 4.6−4.8 (m, 3H, pteridine-CH$_2$ + Glu-α), 6.69 (d, 1H, J = 9.0 Hz, NH), 7.29 (d, 1H, J = 9.0 Hz, benzoxazine-Ar), 7.36 (m, 2H, benzoxazine-Ar), 8.70 (s, 1H, pteridine-Ar). IR (KBr): cm^{-1} 3330, 3190, 1730, 1630, 1560. FAB-MS: m/z 539 (MH$^+$). HR-FAB-MS calcd for $C_{25}H_{31}N_8O_6$: MH$^+$, 539.2366. Found: 539.2379 (MH$^+$).

***N*-[[4-[(2,4-Diaminopteridin-6-yl)methyl]-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl]carbonyl]-L-glutamic Acid (3a).** Using the same procedure as described for the preparation of **3b**, compound **3a** was prepared from compound **21a**. The yield of **3a** was 78% (orange powder). ^{1}H-NMR (DMSO-d_6): δ 1.9−2.0 (m, 2H, Glu-β), 2.2−2.4 (m, 2H, Glu-γ), 3.67 (t, 2H, J = 3.8 Hz, -NCH$_2$-), 4.25 (t, 2H, J = 3.8 Hz, -CH$_2$O-), 4.30 (m, 1H, Glu-α), 4.70 (s, 2H, pteridine-CH$_2$), 6.83 (d, 1H, J = 8.6 Hz, NH), 7.26 (s, 1H, benzoxazine-Ar), 7.30 (d, 1H, J = 8.6 Hz, benzoxazine-Ar), 8.09 (m, 1H, benzoxazine-Ar), 8.70 (s, 1H, pteridine-Ar). IR (KBr): cm^{-1} 3460, 1640, 1510. FAB-MS: m/z 483 (MH$^+$). HR-FAB-MS calcd for $C_{21}H_{23}N_8O_6$: MH$^+$, 483.1740. Found: 483.1737 (MH$^+$). Mp: 203−205 °C dec. Anal. ($C_{21}H_{22}N_8O_6$) C, H, N. Analysis of HPLC (solvent, CH_3CO_2H/CH3CO$_2$Na (pH 5.4):MeOH = 82:18; flow rate, 1.0 cm^3/min; detection, 254 nm) showed the purity to be at least 98% (retention time, 15 min).

2-Aminobenzothiazole-6-carboxylic Acid Hydrochloride (11). To a solution of sodium thiocyanate (880 g, 10.9 mol) and ethyl 4-aminobenzoate (**10**) (1650 g, 10 mol) in methanol (5.1 L) was added bromine (823 g, 5.0 mol) with stirring below −5 °C during the addition, and the mixture was stirred for 2 h at the same temperature. Precipitated white solids were collected by filtration and washed with water. The obtained solids were suspended in 1 N HCl (4.0 L), refluxed, and filtered with heating. To the filtrate was added concentrated HCl (2.0 L), and this was stored in a cold place to afford white solids, which were collected by filtration and dried *in vacuo* to give **11** (807 g, 32%) as a white powder. ^{1}H-NMR ($CDCl_3$−CD_3OD): δ 6.86 (d, 1H, J = 9 Hz, H^4), 7.7−8.1 (m, 2H, H^5 + H^7). IR (KBr): cm^{-1} 3300−3000, 1700, 1630, 1600. MS: m/z 194 (M$^+$), 169. HR-MS calcd for $C_8H_6N_2O_2S$: M, 194.0150. Found: 194.0131 (M$^+$).

4-Amino-3-mercaptobenzoic Acid Hydrochloride (12). A solution of **11** (400 g, 1.74 mol) and KOH (1.74 kg, 31.1 mol) was refluxed for 4 h and then cooled to room temperature and filtered. To the filtrate was added concentrated HCl (2.3 L) dropwise. The precipitated solids were collected by filtration. The obtained solids were washed with water and suspended in water (6.0 L). To this suspension was added $SnCl_2$ (20 g, 0.11 mol), and the mixture was heated at 70 °C with stirring. To the refluxing mixture was added concentrated HCl, until insoluble solids were dissolved. The mixture was then filtered with Celite, and to the filtrate was added 1 N HCl (4.0 L) again. The resulting aqueous solution was stored in a cold place for 1 day. Precipitated white solids were collected by filtration and dried *in vacuo* to give **12** (246 g, 69%) as a white powder. ^{1}H-NMR (DMSO-d_6): δ 6.59 (d, 1H, J = 8 Hz, H^5), 7.3−7.8 (m, 2H, H^2 + H^6). IR (KBr): cm^{-1} 3360, 1710, 1590. MS: m/z 169 (M$^+$), 168. HR-MS calcd for $C_7H_7NO_2S$: M, 169.0198. Found: 169.0212 (M$^+$).

4-Amino-3-[(chloroethyl)thio]benzoic Acid (13). To a mixture of **12** (334 g, 1.63 mol) and NaOH (195 g, 4.88 mol) in EtOH (2.4 L) and water (500 mL) was added 1-bromo-2-chloroethane (542 mL, 6.5 mol) at room temperature, and the mixture was stirred vigorously for 3.5 h. To the reaction mixture were added AcOEt (3.0 L) and water (2.0 L), and the resulting mixture was adjusted to pH 5.0 with 3 N HCl at 0 °C. The organic layer was separated, dried over Na_2SO_4, filtered, and concentrated to give pure **13** (270 g, 72%) as a colorless syrup. ^{1}H-NMR ($CDCl_3$): δ 3.03 (t, 2H, J = 7.6 Hz, -SCH$_2$-), 3.58 (t, 2H, J = 7.6 Hz, -CH$_2$Cl), 6.78 (d, 1H, J = 8.6 Hz, H^5), 7.76 (m, 1H, H^6), 8.03 (d, 1H, J = 2.0 Hz, H^2). IR (neat): cm^{-1} 3470, 1670, 1610. HR-MS calcd for $C_9H_{10}ClNO_2S$: M, 231.0121. Found: 231.0155 (M$^+$).

Methyl 4-Amino-3-[(chloroethyl)thio]benzoate Hydrochloride (14). To a solution of **13** (270 g, 1.17 mol) in MeOH (5.0 L) was bubbled gaseous HCl until saturation, and the mixture was stirred overnight at room temperature. To the reaction mixture were added AcOEt (100 mL) and a large amount of *n*-Hex. Precipitated solids were collected by filtration and dried *in vacuo* to give **14** (256 g, 78%) as a white powder. ^{1}H-NMR ($CDCl_3$): δ 3.01 (t, 2H, J = 7.2 Hz, -SCH$_2$-), 3.55 (t, 2H, J = 7.2 Hz, -CH$_2$Cl), 3.83 (s, 3H, -OCH$_3$), 6.65 (d, 1H, J = 9.0 Hz, H^5), 7.77 (m, 1H, H^6), 8.05 (d, 1H, J = 2.4 Hz, H^2). IR (KBr): cm^{-1} 3460, 3350, 1690, 1620. MS: m/z 247 (M$^+$ + 2), 245 (M$^+$), 194, 182. HR-MS calcd for $C_{10}H_{12}ClNO_2S$: M, 245.0277. Found: 245.0284 (M$^+$).

Methyl 4-(4′-Tolylsulfonyl)-4-amino-3-[(chloroethyl)-thio]benzoate (15). A mixture of **14** (125 g, 0.51 mol) and 4-toluenesulfonyl chloride (108 g, 0.57 mol) in pyridine (500 mL) was stirred at 60 °C for 5 h. The mixture was poured into aqueous NH_4Cl solution and extracted with AcOEt. The organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue was triturated with *n*-Hex, and precipitated solids were collected by filtration. The obtained solids were dried *in vacuo* to give **15** (189 g, 87%) as a white powder. ^{1}H-NMR ($CDCl_3$): δ 2.33 (s, 3H, toluene-CH$_3$), 2.32 (m, 2H, -SCH$_2$-), 2.82 (m, 2H, -CH$_2$Cl), 3.81 (s, 3H, -OCH$_3$), 7.0−7.4 (m, 3H, toluene-Ar2H + H^5), 7.5−7.9 (m, 4H, toluene-Ar2H + H^2, H^6), 7.9−8.1 (m, 1H, NH). IR (KBr): cm^{-1} 3240, 1720, 1600, 1490. MS: m/z 401 (M$^+$ + 2), 399 (M$^+$), 363, 208, 91. HR-MS calcd for $C_{17}H_{18}ClNO_4S_2$: M, 399.0366. Found: 399.0357 (M$^+$).

Methyl 4-(4′-Tolylsulfonyl)-3,4-dihydro-2*H*-1,4-benzothiazine-7-carboxylate (16). To a solution of **15** (188 g, 0.47 mol) in toluene (4.5 L) was added NaH (25 g, 0.63 mol, 60% in mineral oil). The mixture was stirred at 100 °C for 2 h and cooled to room temperature. The cooled mixture was neutralized by concentrated HCl and concentrated under reduced pressure. The obtained residue was poured into aqueous NH_4Cl solution and extracted with AcOEt. The organic layer was dried over Na_2SO_4, filtered, and concentrated to give a brown crude oil. This oil was recrystallized from *n*-Hex−AcOEt to give **16** (116 g, 68%) as a white powder. ^{1}H-NMR ($CDCl_3$): δ 2.40 (s, 3H, toluene-CH$_3$), 2.89 (t, 2H, J = 5.6 Hz, -CHS$_2$-), 3.89 (s, 3H, -OCH$_3$), 4.03 (t, 2H, J = 5.6 Hz, -NCH$_2$-), 7.22 (d, 2H, J = 8.8 Hz, toluene-Ar), 7.49 (d, 2H, J = 8.8 Hz, toluene-Ar), 7.75 (m, 3H, benzothiazine-Ar). IR (KBr): cm^{-1} 1720, 1600, 1430. MS: m/z 363 (M$^+$), 208, 149, 91. HR-MS calcd for $C_{17}H_{17}NO_4S_2$: M, 363.0599. Found: 363.0588 (M$^+$).

4-(4′-Tolylsulfonyl)-3,4-dihydro-2*H*-1,4-benzothiazine-7-carboxylic Acid (17). A mixture of **16** (114 g, 0.31 mol)

and 1 N NaOH (630 mL) in MeOH (3.4 L) was refluxed for 3 h. The mixture was then cooled to room temperature and concentrated to 100 mL under reduced pressure. The residue was diluted with water (2.0 L). This solution was adjusted to pH 2.0 with 3 N HCl. Precipitates were collected by filtration and dried *in vacuo* to give **17** (107 g, 98%) as a white powder. ^{1}H-NMR ($CDCl_3$): δ 2.41 (s, 3H, toluene-CH_3), 2.90 (t, 2H, J = 6.4 Hz, -CH_2S-), 4.02 (t, 2H, J = 6.4 Hz, -NCH_2-), 7.25 (d, 2H, J = 8.7 Hz, toluene-Ar), 7.52 (d, 2H, J = 8.7 Hz, toluene-Ar), 7.79 (m, 3H, benzothiazine-Ar). IR (KBr): cm^{-1} 3100−2900, 1690, 1600. MS: m/z 349 (M^+), 194, 149, 107, 91. Anal. ($C_{16}H_{15}NO_4S_2$) C, H, N, S.

Dimethyl *N*-[[4-(4′-Tolylsulfonyl)-3,4-dihydro-2*H*-1,4-benzothiazin-7-yl]carbonyl]-L-homoglutamate (18d). A solution of **17** (1.47 g, 4.21 mmol) in thionyl chloride (10 mL) was stirred for 2 h at room temperature and concentrated under reduced pressure. The residue was dissolved with CH_2Cl_2 (15 mL). To this solution was added a solution of dimethyl homoglutamate hydrochloride (1.4 g, 6.22 mmol) in water (15 mL). To this mixture was added K_2CO_3 (3.4 g, 24.6 mmol), and the resulting mixture was vigorously stirred overnight. The organic layer was separated, washed with 1 N HCl, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was chromatographed on silica gel with $CHCl_3$−MeOH (100:1) to give **18d** (2.2 g, 92%) as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 1.6−2.1 (m, 4H, hGlu-β,γ), 2.41 (m, 5H, toluene-CH_3 + hGlu-δ), 2.87 (m, 2H, -CH_2S-), 3.68 (s, 3H, hGluα-OCH_3), 3.79 (s, 3H, hGluδ-OCH_3), 4.00 (m, 2H, -NCH_2-), 4.77 (m, 1H, hGlu-α), 6.78 (d, 1H, J = 7.6 Hz, NH), 7.1−7.3 (m, 3H, toluene-Ar2H + benzothiazine-Ar1H), 7.4−7.6 (m, 3H, toluene-Ar2H + benzothiazine-Ar1H), 7.77 (d, 1H, J = 8.6 Hz, benzothiazine-Ar). IR (neat): cm^{-1} 3600−3300, 1730, 1640, 1600. MS: m/z 520 (M^+), 91. HR-MS calcd for $C_{24}H_{28}N_2O_7S_2$: M, 520.1338. Found: 520.1338 (M^+).

Dimethyl *N*-[(3,4-Dihydro-2*H*-1,4-benzothiazin-7-yl)-carbonyl]-L-homoglutamate (19d). A mixture of **18d** (2.2 g, 4.23 mmol) and anisole (2.2 g, 20.3 mmol) in 30% HBr in acetic acid (25 mL) was stirred for 4 h at room temperature and poured into ether (200 mL). Precipitates were decanted with ether. The residue was suspended with 5% $NaHCO_3$ and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4, filtered, and concentrated to give pure **19d** (700 mg, 45%) as white powder. ^{1}H-NMR ($CDCl_3$): δ 1.6−2.0 (m, 4H, hGlu-β,γ), 2.40 (t, 2H, J = 6.9 Hz, hGlu-δ), 3.03 (m, 2H, -CH_2S-), 3.66 (s, 3H, hGluα-OCH_3), 3.70 (m, 2H, -NCH_2-), 3.77 (s, 3H, hGluδ-OCH_3), 4.77 (m, 1H, hGlu-α), 6.44 (d, 1H, J = 8.1 Hz, NH), 6.54 (d, 1H, J = 8.1 Hz, Ar), 7.38 (m, 1H, Ar), 7.49 (d, 1H, J = 2.4 Hz, Ar). IR (KBr): cm^{-1} 3320, 1720, 1630, 1590. MS: m/z 366 (M^+), 178, 122, 59. HR-MS calcd for $C_{17}H_{22}N_2O_5S$: M, 366.1249. Found: 366.1249 (M^+).

Dimethyl *N*-[[4-[(2,4-Diaminopteridin-6-yl)methyl]-3,4-dihydro-2*H*-1,4-benzothiazin-7-yl]carbonyl]-L-homoglutamate (21d). A mixture of **19d** (640 mg, 1.74 mmol) and **20** (712 mg, 1.80 mmol) in DMA (10 mL) was stirred for 3 days at 70 °C. The mixture was poured into 5% $NaHCO_3$ and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue was chromatographed on silica gel with $CHCl_3$−MeOH (10:1) to give **21d** (500 mg, 53%) as a yellow powder. ^{1}H-NMR ($CDCl_3$−CD_3OD): δ 1.5−2.0 (m, 4H, hGlu-β,γ), 2.36 (t, 2H, J = 6.9 Hz, hGlu-δ), 3.14 (m, 2H, -CH_2S-), 3.67 (s, 3H, hGluα-OCH_3), 3.78 (s, 3H, hGluδ-OCH_3), 3.90 (m, 2H, -NCH_2-), 4.77 (m, 1H, hGlu-α), 5.18 (s, 2H, pteridine-CH_2), 6.58 (d, 1H, J = 7.8 Hz, NH), 6.68 (d, 1H, J = 8.6 Hz, benzothiazine-Ar), 7.41 (m, 1H, benzothiazine-Ar), 7.60 (d, 1H, J = 2.2 Hz, benzothiazine-Ar), 8.74 (s, 1H, pteridine-Ar). IR (KBr): cm^{-1} 3500−3200, 1740, 1630. FAB-MS: m/z 541 (MH^+). HR-FAB-MS calcd for $C_{24}H_{29}N_8O_5S$: MH^+, 541.1981. Found: 541.1989 (MH^+).

***N*-[[4-[(2,4-Diaminopteridin-6-yl)methyl]-3,4-dihydro-2*H*-1,4-benzothiazin-7-yl]carbonyl]-L-homoglutamic Acid (3d).** A mixture of **21d** (500 mg, 0.93 mmol) and 1 N NaOH (2.8 mL) in EtOH (23 mL) was stirred overnight at room temperature and concentrated to 2 mL under reduced pressure. The residue was diluted to 5 mL with water and adjusted to pH 3.7 with 1 N HCl. Precipitated solids were collected by filtration and dried *in vacuo* to give **3d** (360 mg, 76%) as a yellowish orange powder. ^{1}H-NMR (DMSO-d_6): δ 1.7−2.2 (m, 4H, hGlu-β,γ), 2.50 (t, 2H, J = 7.2 Hz, hGlu-δ), 3.46 (m, 2H, -CH_2S-), 4.23 (m, 2H, -NCH_2-), 4.58 (m, 1H, hGlu-α), 5.05 (s, 2H, pteridine-CH_2), 7.09 (d, 1H, J = 8.9 Hz, benzothiazine-Ar), 7.73 (m, 2H, benzothiazine-Ar), 8.49 (d, 1H, J = 7.6 Hz, NH), 8.95 (s, 1H, pteridine-CH_2). IR (KBr): cm^{-1} 3500−3300, 1640, 1590, 1500. FAB-MS: m/z 513 (MH^+). HR-FAB-MS calcd for $C_{22}H_{25}N_8O_5S$: MH^+, 513.1669. Found: 513.1675 (MH^+). Mp: 200−203 °C dec. Anal. ($C_{22}H_{24}N_8O_5S\cdot{}^1/_2H_2O$) C, H, N, S. Analysis of HPLC (solvent, CH_3CO_2H/CH_3CO_2Na (pH 5.4):MeOH = 3:1; flow rate, 1.0 cm^3/min; detection, 254 nm) showed the purity to be at least 99% (retention time, 22 min).

Diethyl *N*-[[4-(4′-Tolylsulfonyl)-3,4-dihydro-2*H*-1,4-benzothiazin-7-yl]carbonyl]-L-glutamate (18c). Using the same procedure as described for the preparation of **18d**, compound **18c** was prepared from compound **17** and diethyl glutamate hydrochloride. The yield of **18c** was 95% (colorless oil). ^{1}H-NMR ($CDCl_3$): δ 1.25 (t, 3H, J = 7.3 Hz, Gluα-CH_2CH_3), 1.31 (t, 3H, J = 6.8 Hz, Gluγ-CH_2CH_3), 1.9−2.6 (m, 7H, Glu-β,γ + toluene-CH_3), 2.87 (m, 2H, -CH_2S-), 4.00 (m, 2H, -NCH_2-), 4.1−4.3 (m, 4H, Gluα, δ-CH_2CH_3), 4.76 (m, 1H, Glu-α), 7.03 (d, 1H, J = 7.3 Hz, NH), 7.23 (2H, d, J = 7.8 Hz, toluene-Ar), 7.52 (m, 4H, toluene-Ar2H + benzothiazine-Ar2H), 7.77 (d, 1H, J = 8.4 Hz, benzothiazine-Ar). IR (neat): cm^{-1} 3370, 2980, 1730, 1650. MS: m/z 535 (M^+ + 1), 176.

Diethyl *N*-[(3,4-Dihydro-2*H*-1,4-benzothiazin-7-yl)carbonyl]-L-glutamate (19c). Using the same procedure as described for the preparation of **19d**, compound **19c** was prepared from compound **18c**. The yield of **19c** was 62%. ^{1}H-NMR ($CDCl_3$): δ 1.23 (t, 3H, J = 6.8 Hz, Gluα-CH_2CH_3), 1.30 (t, 3H, J = 6.8 Hz, Gluδ-CH_2CH_3), 2.0−2.5 (m, 4H, Glu-β,γ), 3.01 (t, 2H, J = 4.9 Hz, -CH_2S-), 3.68 (t, 2H, J = 4.9 Hz, -NCH_2-), 4.11 (m, 2H, Gluα-CH_2CH_3), 4.22 (m, 2H, Gluδ-CH_2CH_3), 4.76 (m, 1H, Glu-α), 6.43 (d, 1H, J = 8.6 Hz, Ar), 6.74 (d, 1H, J = 7.3 Hz, NH), 7.34 (d, 1H, J = 8.1 Hz, Ar), 7.48 (s, 1H, Ar). IR (neat): cm^{-1} 3400, 2980, 1720, 1630, 1600. MS: m/z 380 (M^+), 178.

Diethyl *N*-[[4-[(2,4-Diaminopteridin-6-yl)methyl]-3,4-dihydro-2*H*-1,4-benzothiazin-7-yl]carbonyl]-L-glutamate (21c). Using the same procedure as described for the preparation of **21d**, compound **21c** was prepared from compound **19c**. The yield of **21c** was 60%. ^{1}H-NMR ($CDCl_3$−CD_3OD): δ 1.22 (t, 3H, J = 7.3 Hz, Gluα-CH_2CH_3), 1.29 (t, 3H, J = 7.0 Hz, Gluδ-CH_2CH_3), 2.0−2.3 (m, 2H, Glu-β), 2.4−2.5 (m, 2H, Glu-γ), 3.12 (m, 2H, -CH_2S-), 3.89 (m, 2H, -NCH_2-), 4.10 (m, 2H, Gluα-CH_2CH_3), 4.22 (m, 2H, Gluδ-CH_2CH_3), 4.6−4.8 (m, 3H, pteridine-CH_2 + Glu-α), 6.66 (d, 1H, J = 7.1 Hz, benzothiazine-Ar), 7.11 (d, 1H, J = 7.3 Hz, NH), 7.40 (d, 1H, J = 8.6 Hz, benzothiazine-Ar), 7.58 (d, 1H, J = 1.9 Hz, benzothiazine-Ar), 8.67 (s, 1H, pteridine-Ar). IR (KBr): cm^{-1} 3320, 1730, 1630, 1590. FAB-MS: m/z 555 (MH^+). HR-FAB-MS calcd for $C_{25}H_{31}N_8O_5S$: MH^+, 555.2138. Found: 555.2115 (MH^+).

***N*-[[4-[(2,4-Diaminopteridin-6-yl)methyl]-3,4-dihydro-2*H*-1,4-benzothiazin-7-yl]carbonyl]-L-glutamic Acid (3c).** Using the same procedure as described for the preparation of **3d**, compound **3c** was prepared from compound **21c**. The yield of **3c** was 96%. ^{1}H-NMR (DMSO-d_6): δ 1.8−2.2 (m, 2H, Glu-β), 2.30 (m, 2H, Glu-γ), 3.18 (m, 2H, -CH_2S-), 3.95 (m, 2H, -NCH_2-), 4.37 (m, 1H, Glu-α), 4.76 (s, 2H, pteridine-CH_2), 6.79 (d, 1H, J = 8.8 Hz, benzothiazine-Ar), 7.42 (m, 1H, benzothiazine-Ar), 7.59 (d, 1H, J = 2.0 Hz, benzothiazine-Ar), 8.22 (d, 1H, J = 7.3 Hz, NH), 8.67 (s, 1H, pteridine-Ar). IR (KBr): cm^{-1} 3400, 1640, 1590. FAB-MS: m/z 499 (MH^+). HR-FAB-MS calcd for $C_{21}H_{23}N_8O_5S$: MH^+, 499.1512. Found: 499.1521 (MH^+). Mp: 204−207 °C dec. Anal. Calcd for $C_{21}H_{22}N_8O_5S\cdot 3H_2O$: C, 45.65; H, 5.11; N, 20.28; S, 5.84. Found: C, 45.97; H, 4.82; N, 20.26; S, 6.70. Analysis of HPLC (solvent, CH_3CO_2H/CH_3CO_2Na (pH 5.4):MeOH = 3:1; flow rate, 1.0 cm^3/min; detection, 254 nm) showed the purity to be at least 97% (retention time, 15 min).

Peripheral Blood Mononuclear Cell Culture. PBMC from healthy donors 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 (1×10^5 cells/well) were cultured in 0.2 mL in 96-well microtiter plates (Corning #25870) with phytohemagglutinin (0.3 μg/mL) (PHA; Welcome Foundation Ltd., Dartford, U.K.) for 2 days. [^{3}H]Deoxyuridine (UdR; 1 μCi/well) (Amersham International plc, Buckinghamshire, U.K.) 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 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 collagenase (type I; Sigma Chemical Co.) and 0.15 mg/mL 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 third to sixth passage. SC were resuspended in IMDM medium containing 5% FBS, supplemented with penicillin G and streptomycin. Cells (3×10^3 cells/well) were cultured in 0.2 mL in 96-well microtiter plates (Falcon #3072) for 5 days. [^{3}H]UdR (1 μCi/well) was added for the last 2 days of culture, and the proliferation was assessed by determining [^{3}H]UdR uptake.

Induction of Adjuvant Arthritis. Induction of adjutant arthritis was done as previously reported.[18] Briefly, male rats (Lewis, 6-week-old) were inoculated in the base of the tail with 50 μL of liquid paraffin containing 35 μg of heat-killed *Mycobacterium tuberculosis* H37 Ra (Difco Laboratories, Detroit, MI). Drugs were suspended in 0.1% CMC-Na solution and administered orally five times a week for 3 weeks from the day of adjuvant injection. Each group consisted of five rats. The system described by Trentham et al.[19] 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 joint. Arthritic score was evaluated on days 21−23 when arthritic score reached maximum in the control group. The percentage of suppression is expressed as follows:

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

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

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