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FLUORINE-CONTAINING AMINO ACIDS AND THEIR DERIVATIVES. 7.[1] SYNTHESIS AND ANTITUMOR ACTIVITY OF α- AND γ-SUBSTITUTED METHOTREXATE ANALOGS

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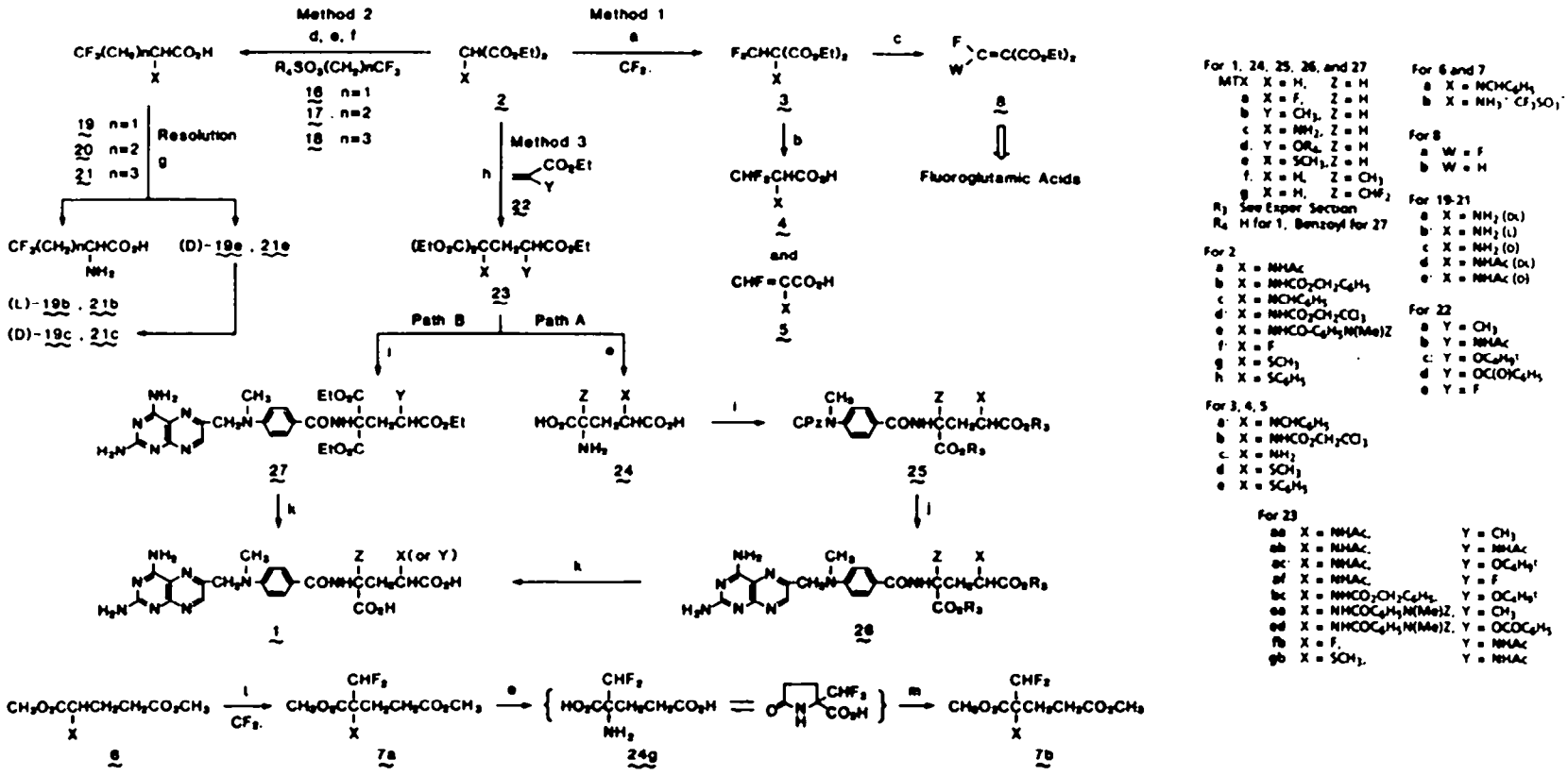
Abstract: Three types of reactions of α-substituted malonates, difluoromethylation, alkylation with n,n,n-trifluoroalkyl sulfonates, and Michael addition to 2-substituted-acrylates, conveniently afforded a number of fluorine-containing α-amino acids such as β-fluorinated-alanines, 2-amino-n,n,n-trifluoroalkanoic acids, and fluorinated glutamic acids as well as other γ-heteroatom-substituted glutamic acids. Here, an efficient enzymatic optical resolution using hog kidney acylase was conducted to obtain both optical isomers of 2-amino-n,n,n-trifluoroalkanoic acids. In addition, a novel sulfoxide rearrangement was observed in a base-catalyzed reaction of diethyl α-difluoromethyl-α-sulfoxy-malonates. Finally, α- and γ-substituted glutamic acids obtained were used for chemical modification of the antitumor agent methotrexate to reveal remarkable structure-activity relationships. In particular, the significant effects of fluorine substitution on the *in vivo* antitumor activity were observed.

Although some naturally occurring halogen-containing amino acids have received considerable attention as chemotherapeutic agents,[2] no fluorine-containing counterparts have been found in nature.[3] Nevertheless, recognition of their wide potential utility has led to the synthesis and biological activity evaluation of diverse types of fluorine-containing amino acids, as has been widely documented.[4] Recently, some β-fluoroamino acids have attracted particular interest owing to their medicinal utility as irreversible or suicide inhibitors of certain amino acid decarboxylases of physiological importance.[5] Given this concrete evidence for their potential utility, fluoro-amino acid chemistry seems to be increasingly penetrating into the field of amino acid and protein chemistry.[6] However, there still remain many synthetic and biochemical challenges. These are, for example, development of synthetic methods for conveniently introducing fluorine into the specific positions on amino acids[7] or for practical asymmetric synthesis,[8] as well as discovery of efficient chemical modification strategies or drug designs[9] by taking advantage of the various unique features of the fluorine atom.

Our previous studies[10] in this field covered the synthesis, conformational analysis, and evaluation of biological activitites of *erythro*- and *threo*-3-fluorophenylalanine,[10a] their derivatives such as 1-fluoro-dehydroxylated chloramphenicol analogs[10b] and N-acylated 3-fluorophenylalanine esters,[10c] α-difluoro-methyl-glutamic acid,[10d] and 3,3-difluoroalanine.[10e] Continuing our work, we did the present study on the synthesis and evaluation of antitumor activity of α- and γ-substituted methotrexate analogs on the basis of the following modification strategy.

Chemical modification of the antitumor agent methotrexate (MTX) remains important in the search for more clinically useful analogs to treat cancer patients, particularly those displaying drug resistance.[11] A recent modification strategy was directed toward the glutamic acid moiety in order to develop less toxic analogs for use in high-dose treatment.[11] A few studies have already been attempted based on the idea that acidity enhancement of the γ-carboxylic acid group might diminish the *in vivo* polyglutamate formation and hence lower its toxicity.[12] We thought that introduction of the most electronegative element, fluorine, into



Scheme 1. (a) $CHClF_2$, $NaN(SiMe_3)_2$ / THF (b) 3N-HCl, 80° (c) Et_3N / CCl_4, r.t. (d) t-BuOK, Sulfonates / THF, refl 1 day (e) Conc HCl, refl 1 day (f) Ac_2O / aq NaOH (g) Acylase / AcOLi, pH 7.0 (h) Na / EtOH, r.t. (i) $SOCl_2$ / ROH, (4)-ZN(CH_3)-C_6H_5COCl-Et_3N/DME (j) HBr / AcOH, DBPH / DMA (k) 0.17-NaOH / 5 : 1 EtOH-H_2O (l) $CHClF_2$-NaF / THF (m) CF_3SO_3H-$SOCl_2$ / MeOH.

this moiety, should significantly enhance the acidity of the carboxylic acid group and hence diminish the toxicity of MTX.[10d] In this full paper, we wish to report the synthesis of MTX analogs, **1a-1g**, containing various γ-substituted glutamic acids such as fluoro-, methyl-, amino-, hydroxyl-, and methylthio-substituted ones as well as two α-substituted ones and the evaluation of their *in vitro* antifolate and *in vivo* antitumor activities. One of the principal objectives of this study was the synthesis of α-amino acids, particularly fluorine-containing ones, for the modification of biologically active amino acid derivatives. Another objective was to find the substituent effects of glutamic acid on the antitumor activity of MTX. This biological portion of the study clearly showed how nonnatural amino acids, like fluorine-containing ones, can be used to modify biologically active amino acid derivatives to shed light on their structure-activity relationships or action mechanisms.

RESULTS AND DISCUSSION

Chemistry

As summarized in Scheme 1 and Table 1, amino acids were prepared in this study by three different reactions of diethyl α-substituted-malonates **2**, namely difluoromethylation (Method 1), alkylation with n,n,n-trifluoroalkyl sulfonates (Method 2), and Michael addition to ethyl 2-substituted-acrylates (Method 3). Since these synthetic methods have many literature precedents[13], only the novel observations are described. For descriptive convenience, these syntheses are separated into two parts, one for fluorine-containing amino acids and the other for other heteroatom-substituted ones.

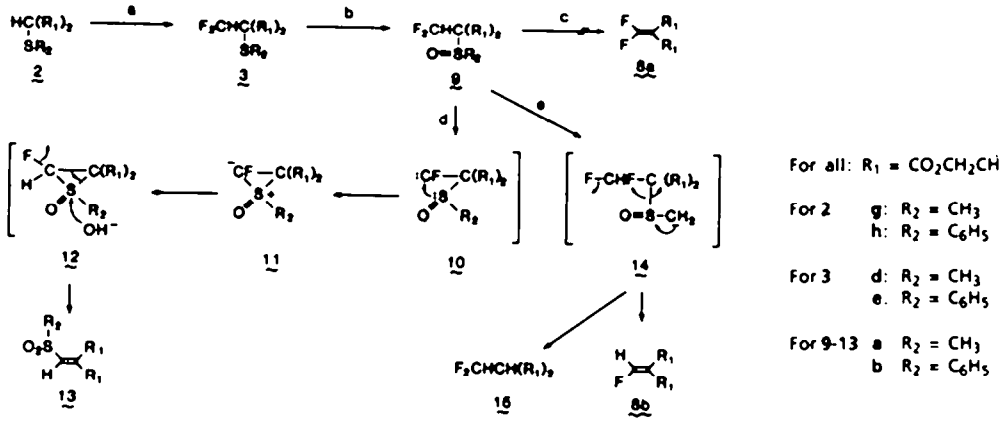
Preparation of Fluoroamino Acids

We have previously reported on the synthesis of β-fluorinated alanines (**4** and **5**)[10e] and α-difluoromethyl glutamic acid (**7b** and **24g**)[10d] via fluorohalomethylation of amino malonates, **2c** and **2d**, and the Schiff base of dimethyl glutamate **6a**, respectively. Along with this line, the reaction of sulfur-substituted malonates, **2g** and **2h**, with difluorocarbene was studied this time in an attempt to prepare a versatile synthon, diethyl difluoromethylenemalonate **8a**, for the synthesis of some other fluoroglutamic acid derivatives as shown in Scheme 1. Upon treatment with potassium tert-butoxide, the carbanions generated from **2g** and **2h** reacted with difluorocarbene at 10° to give the desired difluoromethylated products (**3d** and **3e**) in 42% and 19% yields, respectively. Here, the yield of the reaction significantly decreased in going from **2g** to **2h**. Replacement of the malonate counter cation of potassium with sodium also lowered the yield of the reaction from 42% to 25% in the case of **2g**. The diminished nucleophilicity of the carbanion probably lowered the yield of the reaction. The resulting products **3d** and **3e** were then oxidized to the corresponding sulfoxides **9a** and **9b** in high yields, respectively (See Scheme 2). Unfortunately, the attempt to obtain diethyl difluoromethylenemalonate **8a**[14] from their thermal cis elimination failed, probably because of the significantly decreased acidity of the β-carbon atom by incorporation of two fluorine atoms. Treatment of both sulfoxides with triethylamine at 0° in dichloromethane gave unexpected products, defluorinated and sulfur-migrated vinylsulfones **13a** and **13b** in good yields. The structure of the sulfone **13a** was identified by comparison of its spectral data with those of the authentic sample prepared by addition-elimination of

Table 1. Methods and overall yields for amino acids obtained

Compound	Yield (%)	Method	Compound	Yield (%)	Method
$F_2CHCH(NH_2)CO_2H$ (**4c**)	36	1	$CH_3O_2CCH_2CH_2C(CHF_2)(NH_3{\cdot}OTf)CO_2CH_3$ (**7b**)	38	1
$FCH=C(NHCO_2CH_2CCl_3)CO_2H$ (**5b**)	23	1	$HO_2CCH(F)CH(NH_2)CO_2H$ (**24a**)[a]	70	3A
$CF_3CH_2CH(NH_2)CO_2H$ (**19a**)	36	2	$HO_2CCH(NH_2)CH(NH_2)CO_2H$ (**24c**)	63	3A
$CF_3CH_2CH_2CH(NH_2)CO_2H$ (**20a**)	46	2	$HO_2CCH(SCH_3)CH(NH_2)CO_2H$ (**24e**)	42	3A
$CF_3CH_2CH_2CH_2CH(NH_2)CO_2H$ (**21a**)	49	2			

[a] The alternative method using diethyl α-fluoromalonate as a starting material showed a lower yield of 52% (see also ref 34 and 35).



Scheme 2. (a) $CHClF_2$, Base/THF. (b) m-CPBA/H_2Cl_2. (c) Refl in Xylene-Norbornadiene. (d) Et_3N/CH_2Cl_2, 0° (e) Et_3N/CCl_4, r t

methylmercaptan to diethyl chloromethylenemalonate followed by oxidation with m-chloroperbenzoic acid. As this reaction appears to be rather new and of mechanistic interest, we propose here a possible mechanistic rationale invoking two successively formed carbene and three-membered cyclic sulfoxonium ylide intermediates, 10 and 11, as shown in Scheme 2. In this peculiar sulfoxide rearrangement, the ylide intermediate 11 would be collapsed by protonation and nucleophilic attack by water to give the vinylsulfones, 13a and 13b. Here, a change of reaction conditions, e.g., higher temperature and the use of carbon tetrachloride instead of dichloromethane, led to the formation of two additional products, diethyl fluoromethylenemalonate 8b[15] as the major one and diethyl difluoromethylmalonate 15 as a minor one, besides a small amount of the original sulfone 13. They may have been competitively formed from the intermediate carbanion 14 as depicted in Scheme 2. Here, as 8b was fragile on aqueous work-up, its isolation from the reaction mixture was unsuccessful. We finally abandoned this route for trying to prepare some other fluorine-containing glutamic acid derivatives.

We next found a convenient synthesis and method of optical resolution of a few 2-amino-n,n,n-trifluoroalkanoic acids 19,[16] 20, and 21.[17] Previously, the preparation of these amino acids, particularly, the former two, has required rather tedious procedures. In our method, the carbanion generated from diethyl N-acetylaminomalonate 2a in THF was treated with 2,2,2-trifluoroethyl trifluoromethanesulfonate 16-OTf (abbreviated as triflate hereafter). Trifluoroethylation proceeded smoothly to give the desired diethyl α-(N-acetylamino)-α-(2,2,2-trifluoroethyl)malonate in the isolation yield of 46.1% (see Table 1). However, the reaction did not proceed with the (p)-toluenesulfonate (abbreviated as tosylate hereafter) or with the iodide. Hydrolysis and decarboxylation of the alkylation product easily afforded 2-amino-4,4,4-trifluorobutanoic acid in 36% overall yield. The same reaction was tried with one methylene elongated 3,3,3-trifluoropropyl tosylate in either DMF or THF but only resulted in the formation of complex products including a small amount of the desired product. Possibly, the malonate base might have caused decomposition of the tosylate by abstraction of the β-hydrogen. In any event, elongation of one methylene chain was insufficient to overcome the strong inductive effect of the trifluoromethyl group. Thus, the reaction was examined again with the triflate. The reaction proceeded smoothly to afford the desired alkylated product in 54% yield. This was converted into 20a in 46% overall yield. Elongation of two methylene chains, on the other hand, altered the reaction, allowing it to easily take place with 4,4,4-trifluorobutyl tosylate 18 and thus 2-amino-6,6,6-trifluorohexanoic acid was conveniently prepared in a yield of 49%. In this case, the iodide has been known to afford the same amino acid in 35% yield.[4a]

For optical resolution of these fluorine-containing amino acids,[18] enzymatic resolution using hog-kidney acylase[19] was taken with their N-acetylated derivatives, 19d and 21d. The resolution was highly efficient and both L- and D-isomers were obtained in excellent chemical and optical yields as summarized in Table 2. An exceptional case was hexafluorovaline which totally resisted the enzymatic resolution, as was previously observed also.[6f] Despite this limitation, we consider that the present route can be used as a convenient and practical method for the synthesis of optically active 2-amino-n,n,n-trifluoroalkanoic acids. It should also be possible to use 2,2,2-trifluoroethyl- and 3,3,3-trifluoropropyl triflates, 16-OTf and 17-OTf,

Table 2. Enzymatic optical resolution of 2-amino-n,n,n-trifluoroalkanoic acids

Compound	Separation yield (%) (L)	(D)	$[\alpha]_D$ (c, 4 N HCl) (L)	(D)	HPLC analysis[c] Ret. time (min.) (L)	(D)	Eluent[d] (Vol. %)	Flow rate (ml/min)
19d	97	92	+5.1 ± 0.4[a] (c 1.00)	−4.9 ± 0.4 (c 1.02)	5.49	5.64	90/10	1.0/1
21d	98	97	+18.3 ± 0.6 (c 1.02)	−16.6 ± 0.6 (c 1.02)	11.01	7.64	90/10	1.0/1
ATFL	93	94	+13.2 ± 0.5[b] (c 1.00)	−14.7 ± 0.5[b] (c 1.01)	8.72	8.05	80/20	1.0/1
AHFV	Not separated							

[a] The (L)-enantiomer was obtained previously by F. Weygand et al., and showed: $[\alpha]^{25}_{546}$ −6.3° (c 16.4, 1 N HCl); much lower m.p. 189.5-190.5°. See: W. Steglich, H.-U. Heininger, H. Dworschak, and F. Weygand, *Angew. Chem. Int. Ed. Engl.*, 1967, 6, 807.

[b] These values are for the mixtures of (4*R*)- and (4*S*)-diastereomers. For comple separation of these isomers: See T. Taguchi, A. Kawara, S. Watanabe, Y. Oki, H. Fukushima, Y. Kobayashi, M. Okada, K. Ohta, and Y. Iitaka, *Tetrahedron Lett.*, 1986, 27, 5117 and references cited therein.

[c] These enantiomers separated were determined to be optically pure using the technique of a chiral solvent-generated phase. See ref. 33.

[d] The eluent system used is 0.5 mM $Cu(OAc)_2$-1 mM (L)-Phe/MeOH at pH 4.5 and the column 150 mm x 4.6 mmϕ Nucleosil $_5C_{18}$.

as an alkylating agent in the α-amino acids syntheses which involve the alkylation of glycinates as a key step.[8e-h] At present, we are conducting a study on the use of these fluorine-containing amino acids to modify biologically active oligopeptides; the results will be reported in the near future.

Preparation of γ- and α-Substituted Glutamic Acids

A variety of γ-heteroatom-substituted glutamic acid derivatives such as methyl-, amino-, hydroxyl-, fluoro-, and methylthio-substituted ones **23** were synthesized either by Michael addition of diethyl α-(N-acylamino)malonates, **2a** and **2e**, to ethyl 2-substituted-acrylates **22** or Michael addition of diethyl α-substituted-malonates **2** to ethyl 2-(N-acetylamino)acrylate **22b** as shown by Method 3 in Scheme 1. The former procedure was applied for the synthesis of the adducts **23**-(**aa**, **af**, **bc**, **ea**, and **ed**), whereas the latter was for the adducts **23**-(**ab**, **fb**, and **gb**). Here, an interesting observation was made in the preparation of γ-hydroxyl-substituted glutamic acid derivative **23ac**. Previously, this compound was prepared by the reaction of ethyl 2-t-butyloxy-3-chloropropanoate with **2a**.[20] We found that when the reaction was carried out at higher temperature, it proceeded, though in low yield, as a Michael reaction of ethyl 2-t-butyloxyacrylate **22c** which was formed by the abstraction of hydrogen chloride from t-butyloxy-3-chloropropanoate (see for details the Experimental Section). Therefore, we speculated that α-acyloxyacrylates such as **22d**, which could be easily derived from ethyl pyruvate would more easily undergo Michael reactions. As expected, the reaction with **2e** proceeded smoothly under much milder conditions to produce the adduct **23ed** in 70% yield. Here, as enol pyruvates are usually known as nucleophiles in biogenetic reactions, this reaction is a contrast to these biogenetic ones and may be of synthetic use.[21] On the other hand, as for its nitrogen counterpart, ethyl 2-(N-acetylamino)acrylate[22] has been well known as a common Michael acceptor and is used in this work as well. Therefore, these two cases may clearly indicate that if properly masked by acylation, acrylates with electron donating α-substituents can be easily converted into Michael acceptors. It is also noteworthy that despite both the electron-donating effect to β-carbons (in this case, the γ-carbon) and the α-carbanion destabilizing effect of the olefin-attached fluorine atom, ethyl α-fluoroacrylate **22e**[23] acted as an efficient Michael acceptor to produce the adduct **23af** in a good yield, as previously reported by Hudlicky[35] (see also the Experimental Section). All these adducts **23** heretofore prepared were isolated either as primarily formed linear products or secondary formed cyclic ones. They were all smoothly converted to the desired γ-substituted glutamic acids **24**-(**a**, **c**, and **e**) by acidic hydrolysis followed by decarboxylation.

Meanwhile, (dl)-α-methylglutamic acid **24f** was commercially available and used straightforward for the subsequent reactions. α-Difluoromethylglutamic acid **24g** was prepared by treatment of Schiff base **6** with difluorocarbene followed by acidic hydrolysis as previously reported.[10d] However, it was obtained only as a mixture with its cyclized derivative. In order to exclusively convert it to noncyclized ester **7b**, the use of a very strong acid like trifluoromethanesulfonic acid was required to prevent intramolecular cyclization, probably because of the markedly reduced basicity of the amino group. These glutamic acids obtained were used for the modification of MTX as described below.

Preparation of Methotrexate Analogs

By the essentially same method as that of Piper and Montgomery,[24] methotrexate analogs 1-(a, c, e, f, and g) bearing the γ-fluoro, γ-amino, γ-thiomethyl, α-methyl, and α-difluoromethyl substituents, respectively, were prepared starting from the corresponding free glutamic acids; namely, in sequence by esterification, N-benzoylation, condensation with 2,4-diamino-6-(bromomethyl)-pteridine, and alkaline hydrolysis (see Path A in Scheme 1) (for experimental details: see the Experimental Section).[10d] Analogs 1b and 1d bearing the γ-methyl and the γ-hydroxyl group, respectively, were prepared in another way. Here, the Michael adducts, **23ea** and **23ed**, were directly converted, without hydrolysis to free amino acids as in Path A, to the precursor esters, **27b** and **27d**, by deprotection of the amino group followed by condensation with 2,4-diamino-6-(bromomethyl)pteridine. Mild alkaline hydrolysis of these esters followed by decarboxylation led to the final products, 1b and 1d, bearing the glutamic acid moiety. This route has some advantages over Path A. First, it makes the sequence somewhat shorter by using the substrate, 4-[N-(benzyloxycarbonyl)-methylamino]benzoyl chloride, as a condensation substrate as well as an amino-protecting group. Second, if it is applied to prepare the amino-substituted analog, 1c, the troublesome problem of the double acylation encountered in Path A can be circumvented.

From both Path A and B, the final γ-substituted products 1-(a, b, d, and e) were obtained as a mixture of four diastereomeric and enantiomeric isomers but they could not be seperated. In our initial attempts, separation of diastereomers eventually went well and two pure precursor esters were obtained in the amino and the thiomethyl cases, **26c** and **26e**, as described in Experimental Section. However, in the subsequent hydrolysis, they did not give the corresponding pure free acids, 1c and 1e, because with the thiomethyl case, a very facile epimerization took place on the γ- carbon to produce an equilibrated mixture of the product 1e, and with the amino case, an intramolecular cyclization occurred between the γ-amino and α-carboxylic group to yield a pyrrolidone derivative. With other cases also, complete seperation of the two diastereomers was very difficult and only partially seperated specimens of the methyl- and hydroxyl-substituted derivatives were obtained at the final stage (see Experimental Section). Thus, the *in vitro* antifolate activity and the *in vivo* antitumor activity were evaluated with these partially separated specimens.

Unlike γ-substituted products, α-substituted products 1-(f and g) were obtained only as an enantiomeric mixture[10d] and thus evaluated without separation for these activities.

Biology

All analogs prepared, except 1c, were evaluated for the *in vitro* dihydrofolate reductase inhibitory activities[25] and the *in vivo* antitumor activities[26] against various types of tumors in mice. In the former assay, only free acids were used, whereas in the latter case, either free acids or esters were used, because they usually showed almost the same activities. These results are shown in Table 3 and 4 and discussed below.

In Vitro Antifolate Activity

A spectroscopic enzyme inhibition assay was performed in order to determine the relative binding affinities of these methotrexate analogs with two kinds of dihydrofolate reductases originating from chicken and bovine. The conventional method employed for this purpose is described in the Experimental Section.

Table 3. Dihydrofolate reductase inhibitory activity

Inhibitor (0.03 μM)	Inhibitory activity[a] Bovine liver	Chicken liver
L-MTX	0.50[b]	0.50
1a (F)	0.46 ± 0.03	0.48 ± 0.07
1e (SCH_3)[c]	0.44 ± 0.02	0.38 ± 0.10
1d (OH)[d]	0.41 ± 0.08	0.39 ± 0.07
1d (OH)[e]	0.41 ± 0.04	0.36 ± 0.04
1b (CH_3)[f]	0.37 ± 0.04	0.41 ± 0.07
1b (CH_3)[g]	0.36 ± 0.04	0.34 ± 0.05
1g (α-CH_3)	0.36 ± 0.05	0.38 ± 0.06
1f (α-CHF_2)	0.39 ± 0.01	0.34 ± 0.04

[a] The values are shown as mean ± S.D (n = 3).
[b] Value 0.50 means 50% inhibition of DHFR
[c-g] The diastereomer ratios of these compounds are as follows. [c] 3.7:1; [d] 1.4:1, [e] 1.2:2, [f] 1:1.4; [g] 1:2.1.

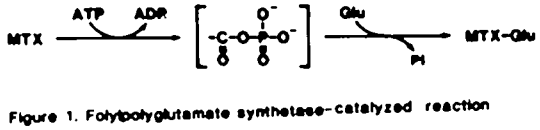


Figure 1. Folylpolyglutamate synthetase-catalyzed reaction

The results obtained are summarized in Table 3. Obviously, all the analogs, 1a-1g, showed a potency similar to that of MTX for inhibiting both DHFRs, although the activities showed a slightly decreasing tendency in going from MTX to other derivatives. These results were significant in the following two aspects. First, they verify, for the first time to our knowledge, a long-standing speculation[27] that enzyme DHFR has considerable tolerance for structural changes in the γ-carboxylic acid group region of the glutamate moiety because the region is located near the surface of the pocket of the enzyme. Secondly, the results suggest that the enzyme may also have tolerance to some extent for structural changes in the α-carbon region of the glutamate moiety.

In Vivo Antitumor Activity

Although the antitumor activities of these analogs were evaluated[28] against several types of tumors in mice, e.g., L1210 and P388 leukemia, Ehlrich carcinoma, B-16 melanoma, and colon 38, the only result with L1210 was summarized here in Table 4. As seen from the data, MTX and its esters produced the almost same excellent increase in life span (ILS value) of 241 and 236%, respectively, at its maximally tolerated dose. On the other hand, γ-substituted derivatives, fluoro 1a, methyl 1b, hydroxyl 1d, and methylthio 1e ones in this order, showed significantly lower ILS, 221% at 640 mg/kg dose, 95% at 40 mg/kg, 79% at 100 mg/kg, 49% at 80 mg/kg, respectively, at their maximally tolerated doses. Here, if we compare these antitumor activities on the basis of the ILS values adjusted to the same molar basis (in this case, 1.9 μmol/kg dose, that means approximately 1 mg/kg), then the order markedly changes to the following one, in decreasing order of activity, probably in correlation with the increase of electronegativity of the γ-substituent: 24% for MTX, 4.7% for the methyl analog 1b, 1.6% for the thiomethyl one 1e, 1.2% for the hydroxyl one 1d, and 0.8% with the fluoro one 1a.[28] Obviously, 1a bearing the most electronegative substituent showed the lowest antitumor activity. Consequently, these results clearly suggested that unlike the *in vitro* antifolate activity, the *in vivo* antitumor activity was significantly affected by the electronegativity of the γ-substituents. Of course, this activity change may have been caused not only by electronic effects but also, to some extent, by steric effects.

As a qualitative index for the activity to toxicity ratio, chemotherapeutic indices (CI) of these analogs were also summarized in Table 4. Interestingly, 1a showed the highest CI value and thus was the least toxic of all the compounds examined. Also, it showed an ILS value almost comparable to that of MTX at its

Table 4. *In vivo* antitumor activity against L-1210 leukemia in mice[a]

Compound (Diastereo. ratio)	Dose[b] (mg/kg/day)	Survival time ILS (%)	Survivers >30 days	CI (ILS_{max}/ILS_{30})	Compound (Diastereo. ratio)	Dose (mg/kg/day)	Survival time ILS (%)	Survivers >30 days	CI (ILS_{max}/ILS_{30})
MTX	1.0	86		50	**1d** (OH)	10	35		8.9
	2.0	119	1		(1 : 2.2)	20	35		
	4.0	171	2			40	45		
	10.0	241	5			80	49		
	20.0	30	1			160	46		
MTX (Isopropyl ester)	1.0	47		19	**1e** (SCH_3) (3.7 : 1)	10	32		>11.1
	4.0	87				40	52		
	10.0	236	4			100	58		
	20.0	>39	3						
1a (F)	1.0	39		71.1	**26e** (SCH_3) (1 : 2.3)	10	23		>6.3
	40	60				20	33		
	160	84				40	49		
	640	>221	3			100	99		
	1280	÷29							
1b (CH_3) (1 : 1.4)	10	57		20	**1f** (α-CH_3)	10	15		
	20	68				40	21		
	40	91				80	23		
	80	88				400	inactive		
	160	21							
1b (CH_3) (1 : 2.1)	10	64		25	**1g** (α-CHF_2)	10	15		
	20	68				40	21		
	40	99				80	23		
	80	39				400	inactive		
	160	37							
1d (OH) (1.4 : 1)	10	23		6					
	20	35							
	40	47							
	80	49							
	160	38							

[a] In each run, seven BDF1 mice (5 weeks, female) were sacrificed to determin the ILS values

[b] The drug was daily administered five times by i.p. injections. The values shown were the daily total amount of injections

maximally tolerated dose. Consequently, **1a** may have some favorable features for high-dose treatment of MTX-resistant cancers.

Unlike γ-substituted derivatives, both α-substituted compounds **1f** and **1g** were very inactive as shown in Table 4. Surprisingly, both the electron-donating methyl and the electron-withdrawing difluoromethyl groups resulted in almost complete loss of the *in vivo* antitumor activity.

Here, we present a brief mechanistic interpretation for these results. Recently, Coward, et al. coincidentary prepared γ-FMTX at the almost same time as we did and showed that it is a potent inhibitor of DHFR but an exceedingly poor substrate for folylpoly(γ-glutamate)synthetase, the enzyme that catalyzes the biosynthesis of the highly retained, cytotoxic MTX polyglutamates and causes meager glutamylation in cells.[29] In addition, they invoked an acylphosphate ion intermediate for this ATP-mediated enzymatic polyglutamation reaction (see Figure 1).[29] These two significant findings indicate that the remarkable substituent effects on the *in vivo* antitumor activity of MTX mainly reflect the substituent effects on the cellular polyglutamation process but not on the inhibitory activity of the target enzyme. The substituent effects on the polyglutamation may have resulted from electronic and/or stereochemical effects on the formation and stability of the acylphosphate ion intermediate involved.

As for the α-substituent effects, the following two speculations can be made. One is their steric retardation of the essential binding of the α-carboxylic group with the enzyme, folylpolyglutamate synthetase. The other is that the α-substitution caused significant changes in chemical and biological properties of the glutamic acid moiety and thus markedly decreased the cellular uptake of the molecules. Further study is needed to examine these speculations.

Recently, the role of polyglutamation of MTX has increasingly come to be recognized as a major determinant of the cytotoxicity and therapeutic selectivity of MTX *in vivo*.[30] The conclusion reached from the present biological studies on these α- and γ-substituted MTX analogs is that γ-FMTX has some interesting antitumor properties despite its greatly reduced *in vivo* activity and thus warrants furthur study.

Experimental Section

M pe were determined with a Yanagimoto hot-stage apparatus and are uncorrected. Unless otherwise stated, ^{1}H- and ^{19}F-NMR were taken on Varian T-60 and EM-360 spectrometers for solutions in $CDCl_3$ containing 1% TMS and 3% hexafluorobenzene as internal standards, respectively, and IR spectra were recorded on a Hitachi 215 grating spectrometer for solutions in $CHCl_3$. Mass spectra were obtained with a Hitachi RMU-6 spectrometer. Elution chromatography was carried out on a Merck Lobar silica-gel column (type B) using chloroform-methanol (20·1) as an eluent. The purity and diastereomer ratios of the final products which were used for biological assays were analyzed by means of HPLC from Waters associates (Model 6000A pump, U6K injector, differential UV detector monitored at 254 nm, and 300 x φ3.9 mm C_{18}-Bondapak column) using a reverse phase system with a mobile phase of 15 % CH_3CN in 0.1 M NaOAc, pH 3.6

Preparation of diethyl α-difluoromethyl-(α-methylthio- and α-phenylthio)malonates, **3d** *and* **3e** Into the well-stirred suspension of potassium tert-butoxide (181 mg, 1 6 mmol) in THF (0.7 ml) was added at -78° under nitrogen a THF solution of **2g** (103 mg, 0.5 mmol/0 7 ml), and the temperature was gradually raised to 10° and maintained for 30 min Next, a THF solution containing chlorodifluoromethane in large excess was added all at once and the mixture was kept well stirred for 30 min at room temperature to complete the reaction The reaction mixture was then poured into 10% aqueous NH_4Cl, saturated with saline, and extracted with EtOAc The organic layer was washed with water three times, dried over $MgSO_4$, filtered, and evaporated *in vacuo*, leaving an oily residue. Chromatography of this residue over Merck silica-gel Lobar column (type B, benzene) afforded 54 mg (42%) of the desired product **3d** as an oily substance. ^{1}H-NMR ($CDCl_3$) δ 1.30 (t, J = 7 0 Hz, 6H), 2.23 (s, 3H), 4 32 (q, J = 7 0 Hz, 4H), 6.32 (t, J_{HF} = 54 6 Hz, 1H); ^{19}F-NMR δ 37 83 (d, J_{HF} = 54 6 Hz, 2F); IR 2960, 2925, 1730, 1310-1170, 1140 cm^{-1}, MS m/z 256 (M^+).

In the same way, the phenylthio derivative **3e** was obtained from **2h** in 19 0% yield· ^{1}H-NMR δ 1 23 (t, J = 7 0 Hz, 6H), 4 17 (q, J = 7.0 Hz, 4H), 6.11 (t, J_{HF} = 53 8 Hz, 1H), 7 17-7 67 (m, 5H); ^{19}F-NMR δ 36 67 (d, J_{HF} = 53 8 Hz, 2F); IR 2980, 1730, 1310-1170, 1145 cm^{-1}, MS m/z 318 (M^+)

Usual oxidation of both **3d** and **3e** with m-CPBA in CH_2Cl_2 at 0° overnight gave the corresponding sulfoxides, **9a** and **9b** As these sulfoxides easily decomposed on silica-gel column chromatography, they were used without purification for the subsequent reactions. Spectroscopic data for **9a**· ^{1}H-NMR δ 1 32 (t, J = 7 0 Hz, 3H), 1.35 (t, J = 7.0 Hz, 3H), 2 86 (s, 3H), 4 33 (q, J = 7.0 Hz, 2H), 4 37 (q, J = 7 0 HZ, 2H), 6 45 (t, 1H), ^{19}F-NMR δ 33 53-35 00 (only four lines observed, 2F); IR 2960, 1730, 1375, 1310-1170, 1095, 1070 cm^{-1}; MS m/z 274 (M^+), For **9b** ^{1}H-NMR δ 0 98 (t, J = 7 0 Hz, 3H), 1 30 (t, J = 7 0Hz, 3H), 3.82 (q, J = 7.0 Hz, 2H), 4.36 (q, J = 7 0 Hz, 2H), 6 42 (t, J_{HF} = 53 7 Hz, 1H), 7 43-8 00 (m, 5H)

Rearrangement of sulfoxides, **9a** *and* **9b**, *to sulfonylmethylene-malonates*, **13a** *and* **13b** The sulfoxide **9a** (31 mg, 0.114 mmol) dissolved in 0.3 ml of CH_2Cl_2 was treated with Et_3N (15 μl, 0 114 mmol) at 0° for 4 h. Next, the reaction was quenched with 10% aq NH_4Cl solution and the products were extracted with CH_2Cl_2 The organic extract was washed with water, dried over MgSO4, filtered, and concentrated *in vacuo*, leaving an oily substance (27 mg, 95%). This was shown to be almost pure by its NMR spectrum and identified as the title compound **13a** by comparison of the spectral data with those of the authentic sample The authentic sample was prepared by the addition-elimination reaction of methylmercaptan to diethyl chloromethylenemalonate followed by oxidation with m-CPBA. Spectral data for **13a**: ^{1}H-NMR δ1.33 (t, J = 7.0 Hz, 2H), 1.35 (t, J = 7 0 Hz, 3H), 3 09 (s, 3H), 4 33 (q, J = 7.0 Hz, 2H), 4.39 (q, J = 7.0 Hz, 2H), 7 32 (s, 1H); IR 2970, 1730, 1440, 1370, 1325, 1280-1170, 1135 cm^{-1}; MS m/z 261 (MH^+).

The phenly-substituted derivative **13b** was also obtained in the same way as **13a**. Spectral data for **13b**: ^{1}H-NMR δ 1.28 (t, J = 7.0 Hz, 3H), 1.41 (t, J = 7.0 Hz, 3H), 4.24 (q, J = 7.0 Hz, 2H), 4.42 (q, J = 7.0 Hz, 2H), 7.19 (s, 1H), 7.40-8.08 (m, 5H); IR 3030, 2970, 1730, 1440, 1370, 1330, 1280-1170, 1150 cm^{-1}; MS m/z 313 (MH^+).

Diethyl fluoromethylenemalonate **8b** *and diethyl difluoromethylmalonate* **15**. **9a** (52 mg, 0.191 mmol) was dissolved in CCl_4 (0.5 ml) at room temperature and treated with Et_3N (26.7 μl, 0.191 mmol) for 2 h. The ^{1}H-NMR spectrum of this solution proved the formation of **8b** as the major product, the structure of which was identified by comparison of its spectral data with those of the authentic sample. The authentic sample was prepared by the addition-elimination reaction of chloromethylenemalonate with KF.[15] The spectrum also confirmed the minor formation of sulfone **13a**. Besides these two products, the ^{19}F-NMR spectrum of this mixture suggested formation of another minor product **15**: ^{19}F-NMR (CCl_4) δ 53.50 (d, J_{HF} = 75.3 Hz, 1H) for **8b** and 39.83 (dd, J_{HF} = 54.6 Hz, J_{HF} = 9.8 Hz, 1H) for **15**. Aqueous work-up of **8b** led to significant loss of fluorine and hence its isolation was unsuccessful.

n,n,n-Trifluoroalkyl sulfonates (**16**, **17**, *and* **18**). The triflate **16-OTf** was prepared in 61% yield from trifluoroethanol and trifluoromethanesulfonic anhydride in the same way as reported[31] except that the aqueous work-up was not done: b.p. 92.5-93.1°/760 mmHg. The tosylate **17-OTs** was prepared in 45% yield from 3,3,3-trifluoropropyltrimethoxysilane by its m-CPBA oxidation followed by tosylation of the resulting alocohol[32]. ^{1}H-NMR δ 2.25-2.73 (m, 2H), 2.47 (s, 3H), 4.20 (t, J = 6.6 Hz, 2H), 7.35 (d, J = 8.0 Hz, 2H), 7.76 (d, J = 8.0 Hz, 2H); IR 1370, 1260, 1195-1140 cm^{-1}. The triflate **17-OTf** was prepared in the same way as **16-OTf**: ^{1}H-NMR δ 2.45-2.90 (m, 2H), 4.70 (t, J = 5.5 Hz, 2H). The tosylate **18-OTs** was prepared in approximately 30% overall yield by a rather lengthy route which involved, in sequence, trifluoroethylation of diethyl malonate with **16-OTf** (61%), alkaline hydrolysis, decarboxylation in 1 N aq. HCl (70%), esterification with diphenyldiazomethane, reduction with $LiAlH_4$ (83%), and tosylation with tosyl chloride (85%). ^{1}H-NMR δ 1.7-2.3 (m, 4H), 2.43 (s, 3H), 4.07 (t, J = 5.0 Hz, 2H), 7.33 (d, J = 8 Hz, 2H), 7.77 (d, J = 8.0 Hz, 2H).

(DL)-2-Amino-4,4,4-trifluorobutanoic acid **19a** *and its N-acetyl derivative* **19d**. **2a** (45.3 g, 208.5 mmol) was dissolved in 450 ml of anhydrous THF under N_2 and treated with t-BuOK (23.4 g, 208.5 mmol) with vigorous stirring at room temperature. After heating at 60° for 2 h, **16** (50.8 g, 218.9 mmol) was added to the resulting suspension of diethyl potassiummalonate in one portion and refluxed for 2 days. Next, it was condensed *in vacuo* to remove most of the THF, quenched with dilute aq. HCl, and extracted with EtOAc. The organic extract was washed with water (2 ×), dried over $MgSO_4$, filtered, and condensed *in vacuo* to a solid-like residue. This residue was separated by silica-gel column chromatography (two type C Merck Lobar columns connected, 2 : 1 cyclohexane-EtOAc) and gave the desired product, diethyl α-(N-acetylamino)-α-(2,2,2-trifluoroethyl)malonate, as a solid material. Recrystallization from Et_2O-hexane afforded the pure specimen (28.7 g, 46.1%). m.p. 69.5-70.5°. ^{1}H-NMR δ 1.27 (t, J = 7.0 Hz, 6H), 2.05 (s, 3H), 3.34 (q, J = 10.5 Hz, 2H), 4.26 (q, J = 7.0 Hz, 4H), 7.13 (br.s, 1H); MS m/z 299 (M^+). (Found: C, 44.03; H, 5.46; N, 4.83; F, 19.30. Calcd for $C_{11}H_{16}NO_5F_3$: C, 44.15; H, 5.39; N, 4.68; F, 19.05). This (32.19 g, 0.108 mol) was completely hydrolyzed and decarboxylated in 170 ml of conc. HCl under refluxing overnight. The reaction mixture was then concentrated *in vacuo* by aspiration and left a solid residue with no contaminating HCl. **19a** (16.2 g, 96%) was isolated from this residue by conventional ion-exchange resin column chromatography (Dowex 50W-X8, 500 ml; aq. 1 N NH_3). This showed m.p. gradually decompd. >230°. **19a** was acetylated by a usual method to afford **19b** in 89% yield: m.p. 133-134° (Recrystd. from Et_2O). ^{1}H-NMR (CD_3OD) δ 1.97 (s, 3H), 2.42-3.17 (m, 2H), 4.18 (dd, J = 7.5 Hz, J = 5.5 Hz, 1H); MS m/z 199 (M^+).

(DL)-2-Amino-5,5,5-trifluoropentanoic acid (**20a**). Diethyl α-(N-acetylamino)-α-(3,3,3-trifluoropropyl)malonate was prepared from **17-OTf** in 54% yield in the same way as described above except that the reaction was done at room temperature. The compound was characterized as follows: m.p. 87-88° (Recrysted. from hexane-Et_2O); ^{1}H-NMR δ 2.26 (t, J = 7.0 Hz, 6H), 2.05 (s, 3H), 1.70-2.70 (m, 4H), 4.22 (q, J = 7.0 Hz, 4H), 6.73 (br.s, 1H); MS m/z 313 (M^+); (Found: C, 45.75; H, 5.78; N, 4.40; F, 18.42. Calcd for $C_{12}H_{18}NO_5F_3$: C, 46.01; H, 5.79; N, 4.47; F, 18.19). This was similarly converted into **20a** in 46% overall yield. **20a** was characterized as follows: m.p. gradually decompd. >215°. ^{1}H-NMR (D_2O) δ (ext. TMS) 2.45-3.10 (m, 4H), 4.25 (t, J = 6.0 Hz, 1H); MS m/z 172 (MH^+). (Found: C, 35.25; H, 4.68; N, 8.15; F, 33.27. Caled for $C_5H_8NO_2F_3$: C, 35.10; H, 4.71; N, 8.19; F, 33.31).

(DL)-2-Amino-6,6,6-trifluorohexanoic acid **21a** *and its N-acetyl derivative* **21d**. Diethyl α-(N-acetylamino)-α-(4,4,4-trifluorobutyl)malonate was prepared from **18-OTs** in 56.8% yield in the same way as described above except that DMF was used instead of THF. The following spectral data was obtained: 1H-NMR δ 1.25 (t, J = 6.9 Hz, 6H), 1.3-2.5 (m, 6H), 2.03 (s, 3H), 4.23 (q, J = 6.9 Hz, 4H), 6.77 (br s, 1H); MS m/z 327 (M^+). This was similarly converted into 21a: m.p. gradually decomposed >225°. **21a** was then converted to its N acetyl derivative **21d**: m.p. 103-104° (Recrystd. from EtOAc-Et2O); 1H-NMR δ 1.42-2.58 (m, 6H), 2.02 (s, 3H), 4.34-4.49 (m, 1H), MS m/z 227 (M^+). (Found: C, 42.11, H, 5.39; N, 6.14, F, 24.98. Calcd for C8H12NO3. C, 42.29, H, 5.32, N, 6.17; F, 25.09).

(DL)-2-(N-Acetylamino)-4-methyl-5,5,5-trifluoropentanoic acid **ATFL**. This was prepared from commercially available 2-amino-4-methyl-5,5,5-trifluoropentanoic acid (5,5,5-trifluoroleucine:**TFL**): m.p. 115-117°.

N-Acylation of hexafluorovaline **HFV**. **HFV** was first converted to the tosyl salt and then esterified with diphenyldiazomethane as usual. This ester was acetylated in dry CH_2Cl_2 with acetyl chloride in the presence of Et_3N and 4-dimethylaminopyridine to produce the benzhydryl ester of **AHFV** in 53% yield: m.p. 81-82° (Found: C, 55.14; H, 4.13; N, 3.33; F, 26.65. Calcd for $C_{20}H_{17}NO_3F_6$: C, 55.43, H, 3.96; N, 3.23, F, 26.38). The benzhydryl ester was cleaved by CF_3COOH-anisole in CH_2Cl_2 as usual to afford free **AHFV**: m.p. gradually decompd. > 172°. ^{1}H-NMR δ 2.08 (s, 3H), 4.29 (ddq, J = 9.0 Hz, J = 2.5 Hz, 1H), 5.52 (m, 1H).

Optical resolution of fluorine-containing amino acids, **19d**, **21d**, **ATFL**, *and* **AHFV**. The example with **21d** represents the general procedures for optical resolution. **21d** (2.185 g, 9.62 mmol) was dissolved in 180 ml of water, brought to pH 11.6 with 1 N aq. LiOH, and then readjusted to pH 7.1 by use of 10% aq. AcOH. Next, hog kidney acylase (E.C. No. 3.5.1.14, 96.2 mg) was added to this buffered solution. After having exactly adjusted its pH to 7.00 with 0.1% aq. AcOH and/or 0.01 N aq. LiOH, the mixture was incubated for 3.5 h at 36° and then quenched by adding 10% aq. AcOH to bring the pH of the solution to 4.5. The mixture was separated by ion-exchange column chromatography (41 ml of Dowex 50W-X8: 100-200 mesh) using water (300 ml) as eluent first until the eluent became neutral and then 1 N aq. NH_3 to elute the amino acid absorbed. From the latter fractions, only ninhydrin-positive fractions were collected and concentrated below 30° *in vacuo* to obtain the almost pure crystalline product, (L)-enantiomer **21b**. This was simply rinsed with MeOH to obtain an analytically pure specimen (873mg, 98.1%): m.p. gradually decompd. >227°; $[\alpha]^{25}_D$ +18.3° ± 0.6 (c 1.05, 4 N HCl); ^{1}H-NMR (D_2O) δ (ext.TMS) 1.88-2.96 (m, 6H), 4.11-4.28 (m, 1H); MS m/z 186 (MH^+). (Found: C, 38.59; H, 5.33, N, 7.62; F, 30.80. Calcd for $C_6H_{10}NO_2F_3$: C, 38.92; H, 5.44; N, 7.57; F, 30.79). HPLC analysis by means of a chiral solvent-generated phase was conducted as reported[33] to determine the optical purity of **21b**. The **21b** obtained was not contaminated with **21c** and was thus optically pure.

The other (D)-enantiomer **21c** (861 mg, 96.8%) was also obtained as an optically pure form by work-up which involved concentration of the water-eluted fractions *in vacuo*, hydrolysis of the residue in 6 N aq. HCl under refluxing overnight, and isolation of the free amino acid by the same ion exchange column chromatography as with **21a**. Compound **21c** was characterized

as follows: m.p. gradually decompd. >214°; $[\alpha]^{25}_D$ -16.6° ± 0.6 (c 1.02, 4 N HCl). (Found: C, 38.65; H, 5.46; N, 7.65; F, 30.50. Calcd for $C_6H_{10}NO_2F_3$: C, 38.92; H, 5.44; N, 7.57; F, 30.79).

Other optically pure amino acids were also prepared in this way. **19b** was obtained in 97% yield and characterized: m.p. gradually decompd. >230°; $[\alpha]^{24}_D$ +5.1° ± 0.4 (c 1.004, 4 N HCl); ^{1}H-NMR (D_2O) δ (ext.TMS) 3.02-3.70 (m, 2H), 4.49 (dd, J = 6.0 Hz, J = 4.0 Hz, 1H); MS m/z 156 (MH^+) (Found: C, 30.88; H, 3.79; N, 9.09; F, 36.19. Calcd for $C_6H_9NO_2F_3$: C, 30.58; H, 3.85; N, 8.92; F, 36.28). **19c** was obtained in 92% yield and characterized: m.p. gradually decompd. >230°; $[\alpha]^{24}_D$ -4.9° ± 0.4 (c 1.016, 4 N HCl); MS m/z 158 (MH^+). (Found: C, 30.33; H, 3.79; N, 8.92; F, 36.43. Calcd for $C_4H_8NO_2F_3$: C, 30.58; H, 3.85; N, 8.92; F, 36.28) The HPLC retention times of these two isomers were not sufficiently different to allow determination of their optical purities However, their opposite $[\alpha]_D$ values and high resolution yields may suggest that these two diastereomers were also optically pure (L)-**TFL** was obtained in 93% yield and characterized: m.p. gradually decompd. >219°; $[\alpha]^{21.5}_D$ +13.2° ± 0.5 (c 1.00, 4 N HCl); ^{1}H-NMR (D_2O) δ (ext.TMS) 1.66 (d, J = 7.5 Hz, 3H), 2.0-3.28 (m, 3H), 4.24 (dd, J = 8.0 Hz, J = 5.5 Hz, 1H); MS m/z 186 (MH^+) (Found: C, 38.45; H, 5.33; N, 7.61; F, 30.53. Calcd for $C_6H_{10}NO_2F_3$: C, 38.92; H, 5.44; N, 7.57; F, 30.79). (D)-**TFL** was obtained in 94% yield and characterized: m.p. gradually decompd. >211°; $[\alpha]^{21.5}_D$ -14.7° ± 0.5 (c 1.01, 4 N HCl); MS m/z 186 (MH^+); (Found: C, 38.84; H, 5.37; N, 7.52; F, 30.66. Calcd for $C_6H_{10}NO_2F_3$: C, 38.92; H, 5.44; N, 7.57; F, 30.53). The HPLC analysis firmly confirmed they were optically pure.

Optical resolution of **AHFV** did not succeed, giving complete recovery of the starting material. **AHFV** did not interfere with the resolution of N-acetylvaline in its competition experiment.

Preparation of γ-substituted glutamic acids, **24a**, **24c**, and **24e**. The reaction was generally carried out in EtOH at 30-50° overnight in the presence of 0.1 equimolar amount of sodium ethoxide as a catalyst. After usual work-up, adducts were separated by silica-gel column chromatography (toluene-EtOAc mixtures). They were subsequently converted into free amino acids by hydrolysis followed by decarboxylation in conc. HCl under reflux overnight. First, **24a** was prepared by two known methods for comparison, by Michael addition of diethyl fluoromalonate to ethyl 2-acetylaminoacrylate[34] and by Michael addition of diethyl malonate to ethyl 2-fluoroacrylate.[35] The latter method showed a higher yield of the adduct, 87%, than the former, 65%. In addition, as diethyl fluoromalonate is as highly toxic as fluoroacetic acid, we used the latter method in enhanced-scale preparation of **24a**, e.g. 20 g to 100 g. **24a** had m.p. decompd. 192-194° (Lit.[35] m.p. 191-192°). **24c** was prepared similarly in 63% yield: m.p. decompd. >230°. (Found: C, 35.65; H, 6.04; N, 16.45 Calcd for $C_5H_{10}N_2O\cdot0.4H_2O$: C, 35.46, H, 6.43; N, 16.54). For the preparation of **24e**, five times more of the base catalyst was used in the Michael addition reaction and the adduct was obtained as a mixture of linear **23gb** and cyclic pyrrolidone derivatives in a moderate yield of 48% This mixture was converted into **24e** without separation in a total yield of 42%. Thus, the **24e** obtained was confirmed to be an almost 1 : 1 mixture of two diastereomers by its nmr spectrum and used for the subsequent reaction leading to **25e** without purification. For **24e**: ^{1}H-NMR (D_2O) δ (ext.TMS) 2.58 and 2.65 (two s, 3H), 2.65-3.15 (m, 2H), 3.97-4.25 (m, 1H), 4.37-4.70 (m, 1H).

Preparation of Michael adducts, **23ea** *and* **23ed**. The starting material **2e** smoothly reacted with α-methyl acrylate **22a** to afford the desired adduct **23ea** as an oil in 96.4% yield in the almost same way as above described: ^{1}H-NMR δ 1.02-1.37 (m, 12H), 2.20-3.10 (m, 3H), 3.35 (s, 3H), 3.78 -4.43 (m, 6H), 5.19 (s, 2H), 7.36 (s, 5H), 7.38 (d, J = 9.0 Hz, 2H), 7.49 (s, 1H), 7.82 (d, J = 9.0 Hz, 2H); IR 3400, 2960, 1725, 1695, 1655, 1320-1130 cm^{-1}; MS m/z 556 (M^+).

Next, for the preparation of **23ed**, α-benzoyloxyacrylate **22d** was prepared as a starting material in 40% yield by treatment of ethyl pyruvate with benzoyl chloride in the presence of Et_3N (the base was added last): ^{1}H-NMR δ 1.28 (t, J = 7.0 Hz, 3H), 4.27 (q, J = 7.0 Hz, 2H), 5.59 (d, J = 1.8 Hz, 1H), 6.14 (d, J = 1.8 Hz, 1H), 7.33-7.77 (m, 3H); IR 1730, 1650, 1600, 1290-1190 cm^{-1}, MS m/z 220 (M^+). This was made to react with **2e** in the usual procedure and the adduct **23ed** was obtained as an oil in 70% yield: ^{1}H-NMR δ 1.03-1.35 (m, 9H), 2.87-3.32 (m, 2H), 3.93-4.32 (m, 6H), 5.06 (s, 2H), 5.39 (dd, J = 8.1 Hz, J = 4.5 Hz, 1H), 6.26 (s, 1H), 7.13-7.58 (m, 3H), 7.32 (s, 5H), 7.99 (dd, J = 7.5 Hz, J = 1.5 Hz, 2H); IR 3400, 2970, 1730, 1280-1170, MS m/z 529 (M^+) On the other hand, the reaction of **2b** with ethyl 2-t-butyloxy-3-chloropropanoate (BCP) at 80° was examined by NMR spectroscopic measurement and TLC analysis of the reaction mixture recovered at an early stage of the reaction. This revealed that BCP had been largely converted to **22c** before **23bc** was formed. For **22c**: ^{1}H-NMR δ 1.23 (t, J = 9.5 Hz, 3H), 1.35 (s, 9H), 4.20 (q, J = 7.0 Hz, 2H), 5.01 and 6.71 (two s, 2H). Thus, **22c** prepared in advance was allowed to react with **2b** for three days under reflux and the adduct **23bc** was obtained in 48% yield: m.p. 88-90°; ^{1}H-NMR δ 1.09 (s, 9H), 1.23 (t, J = 7.0 Hz, 19H), 2.50-2.90 (m, 2H), 3.92-4.42 (m, 7H), 5.10 (s, 2H), 6.87 (s, 1H), 7.33 (s, 5H); IR 1740, 1490, 1280-1170 cm^{-1}; MS m/z 425 ($M^+ - C_4H_8$). (Found: C, 59.79; H, 7.37; N, 3.00 Calcd for $C_{24}H_{36}NO_9$: C, 59.86, H, 7.33, N, 2.91).

N-[4-{[(2,4-Diamino-6-pteridinyl)methyl]methylamino}benzoyl]-4-fluoro-, -4-amino-, and -4-methylthio-glutamic acids (**1a**, **1c**, *and* **1e**). γ-Fluoroglutamic acid (4 g, 24.2 mmol) was converted into the diisopropyl ester hydrochloride by treatment with thionyl chloride in isopropanol for 15 h under reflux: m.p. 140-143°. This was dissolved in dimethoxyethane and coupled with 4-[N-(benzyloxycarbonyl)methylamino]benzoyl chloride (8.82 g, 29.1 mmol, hereafter abreviated as BMAB) with dropwise addition of Et_3N (8.44 ml, 60.5 mmol) at 0°. The mixture was kept well stirred for 2 h and then poured into cold dilute aq. HCl. The products were extracted with EtOAc and the organic layer was washed, twice each, with cold water, dilute aq. $NaHCO_3$, and water, dried over $MgSO_4$, filtered, and concentrated *in vacuo*, leaving an oily residue. Silica-gel column chromatography of this residue (Merck Lobar column type A, 5 · 1 C_6H_6-EtOAc) gave **25a** (11.19 g, 89%) as an oily substance: ^{1}H-NMR δ 1.10-1.43 (m, 12H), 2.13-2.97 (8m, 2H), 3.33 (s, 3H), 4.47-5.70 (m, 4H), 5.18 (s, 2H), 6.93 (d, J = 8.0 Hz, 2H), 7.32 (s, 5H), 7.23-7.97 (m, 4H), ^{19}F-NMR δ -27.58--30.00 (m, 1F), MS m/z 516 (M^+); IR 1725, 1695, 1660, 1100 cm^{-1} Deprotection of the methylamino group with 30% HBr-AcOH gave the free methylamino derivative in 89%. This was purified by silica-gel column chromatography followed by recrystallization from EtOAc-petroleum ether mixture: m.p. 101-104°. This compound (1.15 g, 3.0 mmol) was dissolved in 11 ml of dimethylacetamido and treated with 2,4-diamino-(6-bromomethyl)pteridine hydrobromide (abbreviated as DBPH: 1.19 g, 3.01 mmol as an 1:1 isopropanol adduct) with vigorous stirring. The mixture was heated at 50-55° for 10 h to complete the reaction Next, water was added to the reaction mixture to facilitate precipitation of the desired condensation product with stirring for 2 h under ice cooling. The precipitate was collected by filtration of the mixture, dissolved again into 400 ml of $CHCl_3$, and washed with aq. 0.3 N NH_3 and water The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*, leaving a solid residue. This was separated by silica-gel column chromatography (Merck Lobar column type B, 100 . 5 $CHCl_3$-MeOH) to give the desired product **26a** (1.36 g, 79.1%): m.p. 168-169° (Recrystd. from CH_3CN); ^{1}H-NMR (d_6-DMSO) δ (ext.TMS) 1.00-1.30 (m, 12H), 2.00-2.63 (m, 2H), 3.20 (s, 3H), 4.33-5.53 (m, 4H), 4.78 (s, 2H), 6.57 (s, 2H), 6.82 (d, J = 9.0 Hz, 2H), 7.70 (d, J = 9.0 Hz, 2H), 7.50 (br.s, 2H), 8.38 (d, 1H), 8.57 (s, 1H), ^{19}F-NMR (d_6-DMSO) -26.92--30.42 (m, 1F); IR 1720, 1640, 1600 cm^{-1}; MS m/z 556 (M^+). (Found: C, 54.71; H, 6.16, N, 19.68; F, 3.22. Calcd for $C_{26}H_{33}N_8O_5F\cdot H_2O$: C, 54.34; H, 6.14; N, 19.50; F, 3.31.) Hydrolysis of **26a** was carried out in the same way as reported by Piper et al.[24] except using the slightly different pH conditions of 3.1-3.2 instead of 4.0, which more efficiently effected the precipitation of the desired free acid **1a**. This product was obtained in 83.4% yield and characterized as follows: m.p. gradually decompd. >190°; ^{1}H-NMR (d_6-DMSO) δ (ext.TMS) 2.00-2.63 (m, 2H), 3.20 (s, 3H), 4.27-5.40 (m, 2H), 4.78 (s, 2H), 6.82 (d, J = 9.0 Hz, 2H), 7.73 (br s, 2H), 8.36 (d, J = 7.5 Hz, 1H), 8.59 (s, 1H); ^{19}F-NMR (d_6-DMSO) δ

-22.00–26.00 (m, 1F); IR (KBr disc) 1640, 1600 cm^{-1} (Found: C, 46.21; H, 5.04; N, 21.47; F, 3.47. Calcd for $C_{30}H_{21}N_8O_8F\cdot2.5H_2O$: C, 46.41; H, 5.06; N, 21.66; F, 3.67). The two diastereomers of 1a could not be separated.

(1c) was prepared from γ-aminoglutamic acid 24c by the almost same procedures as used in 1a. In this case, both diastereomers could be separated at the stage of the intermediate 25c, although their conformations could not be elucidated. These two diastereomerically pure intermediates were converted into 26c in 62% yields, respectively and characterized as follows. For isomer A (this isomer has a larger R_f value than B): m.p. 123–125° (Recrystd. from CH_3CN); ^{1}H-NMR ($CDCl_3$ + CD_3OD) δ (ext.TMS) 1.13–1.37 (m, 12H), 1.77–2.43 (m, 2H), 3.16 (s, 3H), 3.50 (dd, J = 9.6 Hz, J = 3.0 Hz, 1H), 4.71 (s, 2H), 4.73–5.25 (m, 3H), 6.73 (d, J = 9.0 Hz, 2H), 7.71 (d, J = 9.0 Hz, 2H), 8.59 (s, 1H); IR 1725, 1640, 1600, 1290–1160 cm^{-1} (Found: C, 55.34; H, 6.47; N, 22.33. Calcd for $C_{26}H_{35}O_5N_9\cdot0.5H_2O$: C, 55.50; H, 6.45; N, 22.41.). For isomer B: m.p. 121–123° (Recrystd. from CH_3CN-CH_3OH). ^{1}H-NMR ($CDCl_3$ + CD_3OD) δ (ext.TMS) 1.13–1.37 (m, 12H), 1.90–2.57 (m, 2H), 3.16 (s, 3H), 3.53 (dd, J = 8.1 Hz, J = 4.5 Hz, 1H), 4.72 (s, 2H), 4.63–5.22 (m, 13H), 6.73 (d, J = 9.0 Hz, 2H), 7.71 (d, J = 9.0 Hz, 2H), 8.58 (s, 1H). IR ($CHCl_3$) 1725, 1640, 1600, 1290–1160 cm^{-1} (Found: C, 55.28; H, 6.48; N, 22.29. Calcd for $C_{26}H_{35}O_5N_9\cdot0.5H_2O$: C, 55.50; H, 6.45; N, 22.41). As we could not obtain fine crystals of both isomers for X-ray crystallographic analysis, the conformations of both isomers were not elucidated. Hydrolysis of these two precursor esters under basic conditions as used in 1a led to the formation of a pyrrolidone derivative by a very facile intramolecular cyclization between the γ-amino and the α-carboxylic acid group. Neither acidic hydrolysis in dilute aq. HCl nor the use of trimethylsilyl iodide was successful. Thus, these precursor esters were used for *in vivo* antitumor screening. But again intramolecular cyclization took place to a large extent even under the neutral buffer conditions employed for the administration of 26c in the *in vivo* antitumor screening. Although other efforts to obtain 1c had not been pursued, we abandoned evaluation of both the *in vitro* antifolate and the *in vivo* antitumor acitivities of 1c.

The methylthio derivative 1e was prepared in the same way as 1a except that the methyl ester was used instead of the isopropyl one as a carboxylic acid-protecting group. In this case, two partially separated fractions with different diastereomer ratios 2.3 : 1 and 1 : 2.3 were obtained at the stage of 25e, as analyzed by preparative HPLC (Develosil ODS 10 μm packed in a stainless steel column 250 x ϕ20). They were converted into the precursor esters 26e-fraction A and B in 85% yield, respectively and characterized as follows. For 26e-fraction A (with 2.3 : 1 diastereomer ratio): m.p. 134–138°; ^{1}H-NMR ($CDCl_3$ + CD_3OD) δ (ext.TMS) 2.14 (s, 3H), 2.20–2.47 (m, 2H), 3.18 (s, 3H), 3.33 (m, 1H), 3.62 (s, 3H), 3.74 (s, 3H), 4.74 (s, 2H), 4.93 (m, 1H), 6.75 (d, J = 9.0 Hz, 2H), 7.70 (d, J = 9.0 Hz, 2H), 8.59 (s, 1H); IR 1730, 1650, 1605, 1300–1160 cm^{-1} (Found: C, 51.27; H, 5.45; N, 20.90; S, 5.87. Calcd for $C_{23}H_{26}O_6N_8S\cdot0.5H_2O$: C, 51.39; H, 5.44; N, 20.84; S, 5.96). For 26e-fraction B (with 1 : 2.3 diastereomer ratio): m.p. 133–136°; ^{1}H-NMR ($CDCl_3$ + CD_3OD) δ (ext.TMS) 1.97–2.60 (m, 2H), 2.14 (s, 3H), 3.21 (s, 3H), 3.33 (m, 1H), 3.73 (s, 3H), 3.77 (s, 3H), 4.78 (s, 2H), 4.87 (m, 1H), 6.79 (d, J = 9.0 Hz, 2H), 7.74 (d, J = 9.0 Hz, 2H), 8.60 (s, 1H); IR 1730, 1650, 1600, 1300–1160 cm^{-1} (Found: C, 51.54; H, 5.28; N, 21.08; S, 5.77. Calcd for $C_{23}H_{26}N_8O_6S\cdot0.5H_2O$: C, 51.39; H, 5.44; N, 20.84; S, 5.96). The same alkaline hydrolysis of these two esters as above described, however, resulted in complete epimerization at the γ-position to produce diastereomeric mixtures with the identical diastereomer ratio (A:B, diastereomer A has a shorter retention time on HPLC analysis than B) of 3.7 : 1. This mixture was obtained in 82% yield and characterized as follows: m.p. 192–199°; ^{1}H-NMR (d_6-DMSO) δ (ext. TMS) 1.77–2.30 (m, 2H), 2.05 (s, 3H), 3.20 (m, 1H), 3.21 (s, 3H), 4.63 (m, 1H), 4.80 (s, 2H), 6.60 (s, 2H), 6.84 (d, J = 9.0 Hz, 2H), 7.72 (br.s, 2H), 7.74 (d, J = 9.0 Hz, 2H), 8.21 (d, J = 7.5 Hz, 1H), 8.60 (s, 1H); IR (KBr disc) 3600–3100, 1640, 1600 cm^{-1} (Found: C, 47.41; H, 5.21, N, 21.10; S, 5.97. Calcd for $C_{21}H_{24}N_8O_5\cdot2H_2O$: C, 47.01; H, 5.26; N, 20.88; S, 5.98). Therefore, precursor ester, 26e-fraction B, was used for the *in vivo* antitumor activity screening as well as the free acid 1e which was used for the *in vitro* antifolate activity screening as well.

N-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-4-methyl- and -4-hydroxyl-glutamic acids (1b and 1d). After deprotection of the methylamino group of 23ea, the resulting methylamino derivative was coupled with DBPH in the same way as in 1a to obtain the precursor ester 27b in 79.6% yield. The ester had m.p. 178–179.5° (Recrystd. from $CHCl_3$-Et_2O) and gave satisfactory analytical data. This ester was hydrolyzed in the same way as described above except for the conditions of 65° for 30 h. The resulting free acid was then decarboxylated to 1b by heating at 80° for 3 h in aq. HCl solution at pH 1.0. The 1b obtained was found to be a diastereomeric mixture with the isomer A : B ratio of 1 : 1.4 and characterized as follows. m.p. gradually decompd. around 189–194°; ^{1}H-NMR (d_6-DMSO) δ (ext.TMS) 1.10 (d, J = 6.6 Hz, 3H), 1.57–2.63 (m, 3H), 3.21 (s, 3H), 4.37 (m, 1H), 4.80 (s, 2H), 6.81 (d, J = 9.0 Hz, 2H), 7.09 (s, 2H), 7.72 (d, J = 9.0 Hz, 2H), 8.00 (br.s, 2H), 8.18 (d, J = 7.5 Hz, 1H), 8.61 (s, 1H); IR (KBr disc) 3600–3100, 1640, 1600 cm^{-1}. (Found: C, 48.96; H, 5.65; N, 21.52. Calcd for $C_{21}H_{24}N_8O_5\cdot2.5H_2O$: C, 49.12, H, 5.69; N, 21.82). The use of different decarboxylation conditions, e.g. a higher pH value of 4.0, afforded, in one case, a diastereomeric mixture with a different isomer ratio of 1 : 2.1, which had m.p. (decompd.) 190–195° and showed satisfactory analytical data. These two specimens were used for both screenings.

The hydroxyl derivative 1d was prepared from the adduct 23ed via precursor ester 27d (X = $OCOC_6H_5$) in the same way. The ester was obtained in 82.5% yield, had m.p. 125–127° (Recrystd. from $CHCl_3$-Et_2O) and gave satisfactory analytical data. It was hydrolyzed at room temperature and then decarboxylated in the same way as described above to obtain 1d in 77.9% yield. Thus specimen obtained was found to be a diastereomeric mixture of the isomer ratio of 1.4:1 by HPLC analysis and characterized as follows: m.p. gradually decompd. 240–250°; ^{1}H-NMR (d_6-DMSO) 1.73–2.27 (m, 2H), 3.20 (s, 3H), 3.80–5.20 (m, 2H), 4.79 (s, 2H), 6.83 (d, J = 9.0 Hz, 2H), 6.87 (s, 2H), 7.60 (br.s, 2H), 7.73 (d, J = 9.0 Hz, 2H), 8.28 (d, J = 7.5 Hz, 1H), 8.61 (s, 1H); IR 3600–3100, 1630, 1600 cm^{-1} (Found: C, 44.65, H, 5.04, N, 20.40. Calcd for $C_{20}H_{23}N_8O_6\cdot3.5H_2O$: C, 45.03; H, 5.48; N, 21.00.). Like 1b, use of different conditions for decarboxylation afforded a mixture with the different diastereomer ratio of 1 : 2.2, which had m.p. (decompd.) 240–250° and showed satisfactory analytical data. These specimens were used for both screenings.

N-[4-[[2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-2-methyl- and -2-difluoromethyl-glutamic acids (1f and 1g). These two α-substituted MTX analogs were prepared in the same way as 1a and characterized as previously reported.[10d]

Enzyme assay for antifolate activity. Inhibitory effects of these MTX analogs on bovine and chicken liver dihydrofolate reductase were examined *in vitro*. The dihydrofolate reductase activity was assayed by incubating 0.1 mM dihydrofolate, 0.1 mM NADPH, 0.11 mM dithiothreitol, 50 mM KH_2PO_4 (pH 7.4), and the enzyme preparations (Sigma), in a total volume of 3 ml, at 37°. The amount of enzyme used was 5–7 μg bovine enzyme (sp. 6.7 U/mg) and 5–9 μg chicken enzyme (sp. 3.7 U/mg) per 3 ml cuvette. The reaction was initiated by adding NADPH and the change in absorbance at 340 nm was followed for 80 sec with Shimazu UV-300 spectrophotometer to determine the initial rate of the reaction. Since 30 nM MTX inhibited the reaction 50%, inhibitory effect of test compounds was examined at 30 nM concentration and the results were compared with those of MTX (Table 1).

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