Analogues of Methotrexate in Rheumatoid Arthritis. 1. Effects of 10-Deazaaminopterin Analogues on Type II Collagen-Induced Arthritis in Mice

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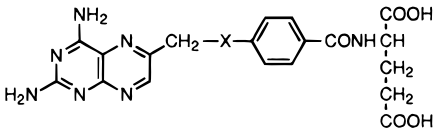
Carbonation of the dianions (LDA) of 5-methylthiophene-2-carboxylic, 2-methylpyridine-5-carboxylic, and 3-methylpyridine-6-carboxylic acids provided the respective carboxy heteroarylacetic acids. The crude diacids were directly esterified in MeOH−HCl to afford the diesters. Alkylation of the sodio anions with ethyl iodide yielded the appropriate α-ethyl diesters. The anions of the various diester substrates were then alkylated by 2,4-diamino-6-(bromomethyl)-pteridine followed by ester saponification at room temperature to afford the respective 2,4-diamino-4-deoxy-10-carboxy-10-deazapteroic acids. The 10-carboxyl group was readily decarboxylated by heating in DMSO at temperatures of 110−135 °C to give the diamino 10-deaza heteropteroic acid intermediates. Coupling with diethyl L-glutamate followed by ester hydrolysis afforded the target aminopterins. The analogues were evaluated for antiinflammatory effect in the mouse type II collagen model. The thiophene analogue of 10-ethyl-10-deazaaminopterin was found to be an effective inhibitor in terms of reduced visual evidence of inflammation and swelling as determined by caliper measurement.

Rheumatoid arthritis (RA) is an inflammation of the joints arising from infectious, metabolic, or autoimmune causes of unknown origin. All forms of arthritis have, as a final manifestation, loss of joint function due to erosion of the articular cartilage surface and subsequent limitation of range of motion due to painful articulation. Onset of cartilage destruction is linked to a common biochemical pathway involving release of cartilage proteoglycans from the extracellular matrix.[1−3] In infectious and inflammatory arthritis, loss of proteoglycan precedes any change in collagen levels[4] and any visually apparent effects. A working hypothesis is that early and effective intervention by a drug that reduces proteoglycan degradation prior to cartilage breakdown will potentially prevent progression of disease and provide for cartilage repair. Nonsteroidal antiinflammatory drugs have been used most frequently to suppress the inflammation of rheumatoid arthritis, but these drugs which inhibit prostaglandin synthesis in chondrocytes, do not interfere with the underlying immune-based processes believed to be paramount in rheumatoid arthritis. Refractory cases have been treated with strongly cytotoxic agents such as azathioprine,[4] chlorambucil,[5] and cyclophosphamide,[6] but these compounds are renowned for carcinogenic properties and adverse drug reactions.

The antifolate drug methotrexate (MTX) has been used as an antitumor agent since its approval in 1955, but in more recent years it has become a prominent if not favored drug for treatment of RA.[7] Several clinical trials[8] have verified the efficacy of MTX for treatment of advanced and refractory cases of RA. In a summation of major results seen with MTX in placebo-controlled clinical trials,[9−12] one observes an improvement in inflamed joint counts of 17−35% and pain decreases of about 26−51% over nonsteroidal antiinflammatory drugs (NSAIDs) alone. MTX is administered by injection or orally at doses of 7−15 mg/patient/week for RA, doses that are considerably below the cancer therapy doses of 50−60 mg/patient/week. Principal acute toxicities occur with liver, kidney, and mucosal tissue particularly in the gastrointestinal tract.[10] Liver toxicity is evidenced[13] by increased levels of SGOT enzyme activity, but these are of a transient nature even at the high doses used in cancer therapy. There has been concern that chronic therapy with MTX in RA could cause liver damage, but this has not been a significant limitation in long-term patients.

The ability of MTX to affect the inflammatory conditions of RA may be linked to its cytotoxic behavior. This is probably in the nature of immune suppression and could involve attack on inflammatory phagocytic cells such as macrophages and neutrophils or subsets of T-helper cells located in the synovial region. Very few MTX analogues have been evaluated in animal models of RA, and there is no clear indication whether the antiarthritic properties are directly proportional to cytotoxicity or immune suppression.



X = NCH_3 Methotrexate (MTX)
X = CH_2 10-Deazaaminopterin (10-DAM)
X = CHEt- Edatrexate (10-Et-10-DAM)

Galivan and co-workers[14] showed that adjuvant arthritis and streptococcal cell wall arthritis in rats responded to doses of MTX relative to those used in man for treatment of RA. They also found that timing of

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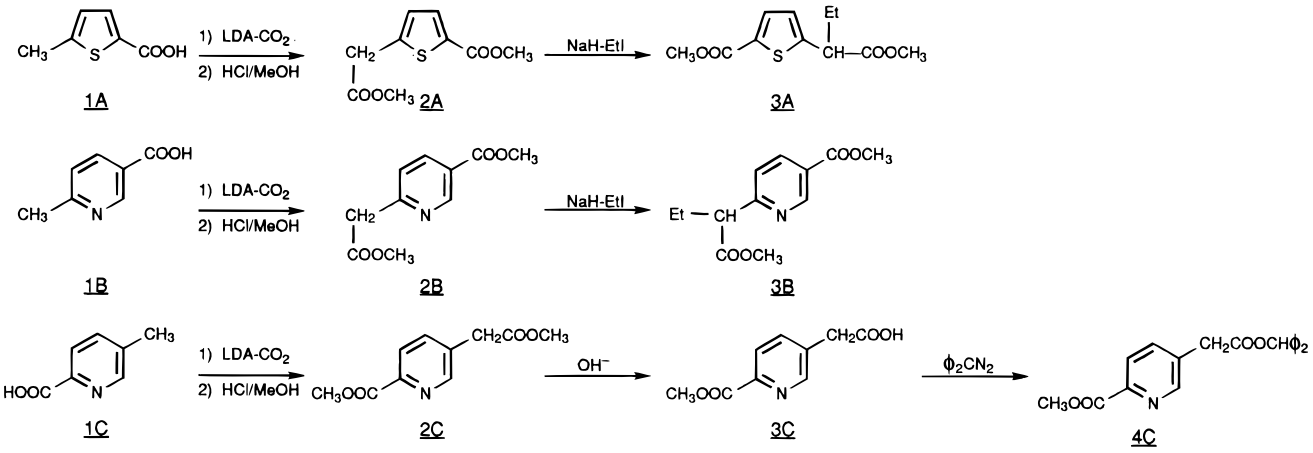
Scheme 1



dosing was most important for reduction of inflammation. MTX and aminopterin were found to inhibit inflammation, but other antifolates lacking a 2,4-diaminopyrimidine moiety or a benzoylglutamate side chain were ineffective. Krumdieck *et al.*[15] have investigated in patients with RA, the activity of 10-deazaaminopterin (10-DAM), an antitumor drug that was developed in our laboratories[16,17] as part of a series of 10-deaza analogues. In a limited clinical study, the compound was found to have an effect comparable to MTX. A comparative study[18] of 10-DAM versus MTX was conducted in a model of spontaneous autoimmune disease in the MRL/1pr mouse. The compounds also had similar effects in this model for skin lesion−proteinuria scores and toxicity.

As part of an extensive program for synthesis and evaluation of new analogues of MTX in rheumatoid arthritis, we report the synthesis and evaluation of some 10-deazaaminopterin compounds featuring replacement of the benzoate moiety with heteroaroyl units.

Chemistry

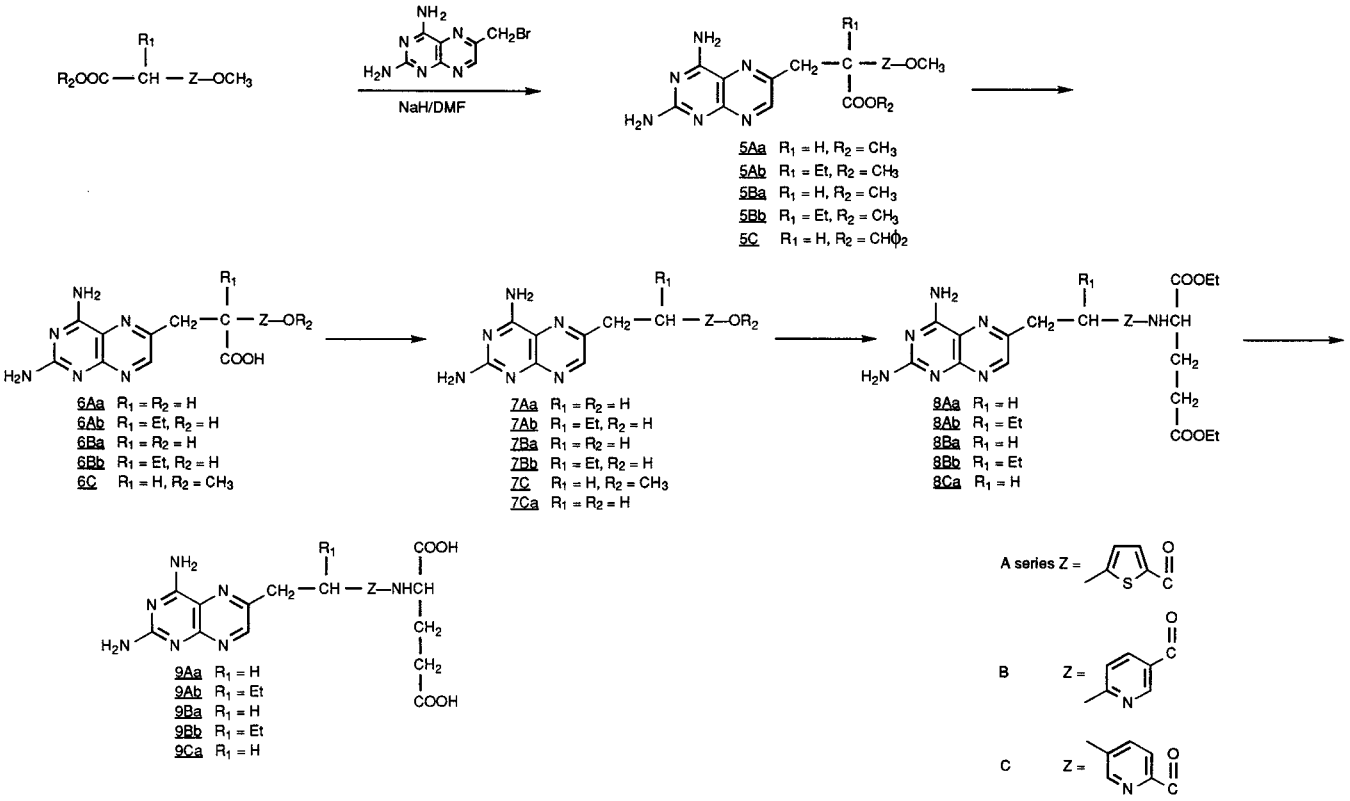
We have previously reported[17] the synthesis of 10-alkyl-10-deazaaminopterin analogues by an abbreviated procedure based on condensation of 2,4,5,6-tetraaminopyrimidine with an α-bromoaldehyde moiety containing the benzoate side chain. However, we have since adopted an alternate process[19] as outlined in Schemes 1 and 2. The key steps in the procedure are alkylation of a suitable (carbomethoxyaryl)acetic acid ester with 2,4-diamino-6-(bromomethyl)pteridine followed by hydrolysis to a 10-carboxypteroic acid intermediate and subsequent facile decarboxylation to the appropriate pteroic acid. We have found the general method to be convenient and adaptable to the synthesis of virtually any 10-deazapterin analogue.

As shown in Scheme 1, 5-methylthiophene-2-carboxylic acid (**1A**) was converted to its dianion by treatment with 2 equiv of lithium diisopropylamide (LDA) in dry THF at −30 °C. The resulting dianion was then treated with gaseous CO_2 to generate the 2-(carboxymethyl)-thiophene-5-carboxylate salt. Following collection of the precipitated salt, the crude material was treated directly with methanolic hydrogen chloride to yield the dimethyl ester **2A** in 51% yield. HPLC of the salt prior to esterification indicated a mixture 78% diacid and 22% starting acid (**1A**). The diester **2A** was converted to its anion with 1 equiv of NaH in DMF and alkylated at −30 °C with 2,4-diamino-6-(bromomethyl)pteridine to yield the 10-carboxypteroate dimethyl ester intermediate (**5Aa**) in 88% yield. The diester was saponified with 1 N NaOH in 2-methoxyethanol at room temperature followed by careful acidification to pH 5 to precipitate the diacid **6Aa** in 77% yield. Compound 6Aa was then dissolved in DMSO and the solution heated at 135 °C for 45 min to effect decarboxylation to the 4-amino-4-deoxypteroic acid thiophene analogue **7Aa** in 84% yield. Precipitation of the 10-COOH diacids (*e.g.*, **6Aa**) at a pH lower than 5 frequently caused the decarboxylation process to proceed with difficulty, possibly because the free 4-NH_2 group may be assisting in the decarboxylation process. The procedure is based upon our previous work[20] in the 8,10-dideazaaminopterin series where decarboxylation was achieved with ester cleavage by prolonged heating with NaCN−DMSO at 180 °C. That method was erratic and usually produced dark-colored, impure products.

The thienylpteroic acid intermediate was coupled with diethyl L-glutamate in the usual manner with activation of the thienyl carboxylate with isobutyl chloroformate in DMF to afford the glutamate diester **8Aa** in 32% yield following purification by chromatography on silica gel. Saponification at room temperature readily yielded the target 10-deazaaminopterin thienyl analogue **9Aa**. The general procedure was also applied to the synthesis of the 10-ethyl analogue **9Ab**. Alkylation of the anion of thiophene diester **2A** with ethyl iodide afforded the α-ethyl diester **3A**, readily separable from any unreacted **2A** by column chromatography on silica gel. Decarboxylation of the 10-ethyl-10-COOH intermediate **6Ab** was even more facile, requiring a temperature of only 125 °C for 30 min.

The 3′-pyridyl analogue (**9Ba**) of 10-deazaaminopterin and its 10-ethyl analogue **9Bb** were prepared in a similar fashion. 6-Methylnicotinic acid (**1B**) was converted to its dianion with LDA, carbonated, and esterified (HCl−MeOH) to give the (5-carboxy-2-pyridyl)acetic acid dimethyl ester (**2B**) in 30% yield after chromatography. Alkylation of **2B** with ethyl iodide afforded the α-ethyl diester **3B** in 51% yield. Decarboxylation of the 10-COOH diacid **6Ba** occurred over a 20 min period at 110 °C, reflecting participation of the adjacent pyridine nitrogen atom. This assistance rendered by the pyridine nitrogen was even more evident in decarboxylation of the α-ethyl 10-COOH diacid **6Bb**. The process took place at room temperature over 20 min in a DMF solution to give **7Bb** in essentially quantitative yield.

Scheme 2



The 2′-pyridyl analogue of 10-deazaaminopterin was synthesized starting from 5-methylpicolinic acid (**1C**). After dianion formation with LDA and carbonation, the dimethyl ester of (2-carboxy-5-pyridyl)acetic acid (**2C**) was obtained in 49% yield via treatment of the diacid salt with HCl−MeOH. Since there was concern that the carboxyl on the pyridine ring would also be lost in the decarboxylation step, we prepared the methyl benzhydryl mixed diester **4C**. Selective alkaline hydrolysis of **2C** gave the ester acid **3C** which was converted to **4C** by esterification with diphenyldiazomethane. Alkylation of **4C** with 2,4-diamino-6-(bromomethyl)pteridine to **5C** and cleavage of the benzhydryl ester with trifluoroacetic acid in $MeCl_2$ afforded the 10-carboxy-2′-pyridylpteroate ester **6C**. Decarboxylation (130 °C in DMSO) gave the pteroate ester **7C** which was saponified at room temperature to the pyridylpteroic acid analogue **7Ca**. Coupling with glutamate and saponification gave the target aminopterin **9Ca**.

Biological Evaluation

The analogues were evaluated both for efficacy in an animal model of rheumatoid arthritis and for their cytotoxicity in cellular systems *in vitro*. The animal model selected is the mouse collagen II-induced arthritis assay described by Courtenay.[21] Methotrexate compounds have been assayed previously by the classical rat adjuvant method. We chose the collagen-induced assay because it represents an inflammatory process that more closely resembles the rheumatoid arthritis condition in humans. In this procedure, DBA/1 mice, a genetically conserved strain, are injected with bovine II collagen to induce disease. In our modification of the Courtenay procedure, a booster dose of the antigen is administered at day 21. Visual evidence of inflammation is observed after about day 23, while measurable swelling is seen by days 23−30. In most runs, 75−100% of the control mice show acute inflammation by day 37. The MTX analogues were applied intraperitoneally 2 days before the initial collagen II challenge, and a dosing schedule of every 2 days was maintained thereafter.

The data presented in Tables 1 and 2 demonstrate that derivatives of methotrexate are functional in alleviating collagen-induced arthritis. The compounds 10-DAM, 10-Et-10-DAM, and **9Aa**,**Ab**,**Ba**,**Ca** decreased the incidence and severity of the arthritis over a variable range of doses. As shown in Table 1, the 10-ethyl-10-deazaaminopterin thienyl analogue **9Ab** was the most effective compound in terms of decreased incidence of disease when compared with MTX. Only 12% of animals treated with **9Ab** showed visual disease even at day 44, while MTX was clearly showing indications of inflammation at day 44. Compound **9Ab** was also quite effective at reduction of swelling in the foot pad at 10−15 mg/kg, while MTX was equally effective at a dose of 9 mg/kg. 10-DAM which has shown clinical efficacy[15] was effective in reduction of swelling, but a significant percentage of animals had visible inflammation. 10-Et-10-DAM was a little more effective than its thienyl counterpart **9Ab** for reduction of swelling. The 2′-pyridyl analogue **9Ca** was apparently very effective at a dose of 12 mg/kg, but this was clearly an unacceptably toxic dose. Unfortunately, the supply of compound and scheduling limitations did not permit further evaluation at lower doses. Compound **9Bb** was ineffective in decreasing onset of disease or severity at the test doses.

We have also measured the general cytotoxicity of the compounds in cell culture with L1210 cells and a representative human liver cell line, namely, the Chang immortalized liver cell.[22] The 3′-pyridyl analogues **9Ba**,**Bb** were substantially less cytotoxic to L1210 cells than MTX by a factor of 4.4 and 13.4, respectively. Both were less cytotoxic than MTX versus the Chang cell line.

Table 1. Effects of 10-Deaza Analogues of Methotrexate on Incidence of Mouse Type II Collagen Arthritis

compd	dose (mg/kg, ip)	onset and incidence of disease[a,b] (% animals) day 23	day 30	day 37	day 44
untreated	0	0	25	75	90
MTX	12	0	12	14[c]	0
	9	0	0	0	25
10-DAM	15	0	38	50	38
	12	12	50	50	62
	9	25	38	38	50
10-Et-10-DAM[f]	18	12	0	12	12
	12	25	25	38	50
9Aa[e]	18	0	0	38	25
	12	0	0	50	50
9Ab	15	0	0	12	12
	10	0	0	25	38
9Ba	8	0	38	25	50
	4	0	57	71	100
	2	0	50	63	88
9Bb	12	12	25	88	100
	5	12	63	75	88
	2.5	0	25	75	75
	1.25	12	38	75	100
9Ca	12	0	0[c]	0[c]	*d*

[a] Incidence of disease was scored as the percentage of animals within each experimental group that exhibited joint or limb inflammation. [b] Untreated animals typically show an incidence of arthritis of 80−100%. [c] One animal died in these groups. [d] Three animals died. [e] Nair and co-workers have also synthesized **9Aa** by a different route and showed it to have antiarthritic properties.[32] [f] 10-Et-10-DAM was also shown to be effective in a rat adjuvant model.[33]

However, the two thienyl analogues **9Aa**,**Ab** were highly cytotoxic in both cell lines.

There is a suggestion in this 10-deazaaminopterin series that cytotoxic potency and antiarthritic activity are related, but the conclusion is limited because of the small number of compounds studied. As stated above the 10-ethyl-3-pyridyl analogue **9Bb** was far less cytotoxic to L1210 cells and significantly less cytotoxic to Chang liver calls than MTX. This compound was clearly ineffective against the collagen-induced arthritis in terms of incidence of disease and reduction of inflammation. Compound **9Ba** was more cytotoxic than **9Bb** to L1210 and fairly active against the arthritis at the highest dose tested. The agents that were highly cytotoxic to L1210 such as 10-DAM, 10-Et-10-DAM, and **9Aa**,**Ab** were considerably more effective than their less cytotoxic counterparts **9Ba**,**Bb**.

Table 3. Cytotoxicity of 10-Deazaaminopterin Analogues in L1210 and Chang Liver Cells[a]

compd	growth inhibition IC_{50} (nM)[b] L1210	Chang liver
9Aa	8.1	3.1
9Ab	3.7	1.6
9Ba	88	36
9Bb	268	35
10-DAM	3.7	98
MTX	20	14

[a] See ref 17 for methods. [b] Average of three runs.

Acute toxicity measurements were carried out in four rodent species for compound **9Ab** vs MTX. The LD_{10} values in DBA mice for **9Ab** and MTX were >15 and 12 mg/kg and in BD_2F mice 12 and 7.5 mg/kg, respectively. In the Lewis rat the comparison was striking with a value of 15 mg/kg for **9Ab** against 1.5 mg/kg for MTX. In CD rats the ratio was 4.0 mg/kg for **9Ab** vs 1.0 mg/kg for MTX. A 90-day semichronic toxicity study in CD rats showed a maximum tolerated dose of 1.5 mg/kg for **9Ab** and 0.75 mg/kg for MTX. All of these assays were conducted with intraperitoneal administration. Except for the Lewis rat case, the toxicity advantages of **9Ab** over MTX are moderate and do not suggest that a major therapeutic advantage would be expected.

The mechanism of action by which methotrexate decreases the onset and severity of mouse type II collagen arthritis is unknown. The disease process in susceptible mice is characterized by high titers of anticollagen antibody, and there are reports that the antibodies include the isotypes IgG_1 and IgG_{2a}, both capable of fixing complement.[23−25] In addition, the collagen arthritic mouse exhibits a T-cell proliferative response to type II collagen, and a delayed-type hypersensitivity reaction occurs in these animals with a peak response prior to onset of arthritis.[26−28] These observa-

Table 2. Antiinflammatory Effects of 10-Deaza Analogues of Methotrexate on Mouse Type II Collagen Arthritis

compd	dose[a] (mg/kg, ip)	paw swelling[b] (mean ± SD, mm) day 23	day 30	day 37	day 44
untreated	0	2.33 ± 0.21	2.43 ± 0.16	3.09 ± 0.91	3.00 ± 0.54
MTX	12[c]	2.27 ± 0.23	2.26 ± 0.17	2.28 ± 0.13	2.22 ± 0.09
	9	2.14 ± 0.04	2.16 ± 0.06	2.18 ± 0.02	2.24 ± 0.13
10-DAM	15	2.20 ± 0.11	2.31 ± 0.11	2.29 ± 0.15	2.30 ± 0.18
	12	2.21 ± 0.12	2.36 ± 0.13	2.31 ± 0.10	2.33 ± 0.13
	9	2.19 ± 0.13	2.32 ± 0.15	2.28 ± 0.14	2.32 ± 0.13
10-Et-10-DAM	18	2.18 ± 0.11	2.24 ± 0.08	2.19 ± 0.12	2.18 ± 0.16
	12	2.26 ± 0.25	2.28 ± 0.18	2.34 ± 0.16	2.32 ± 0.27
9Aa	18	2.14 ± 0.03	2.16 ± 0.03	2.26 ± 0.17	2.38 ± 0.55
	12	2.15 ± 0.04	2.15 ± 0.05	2.28 ± 0.18	2.58 ± 0.74
9Ab	15	2.15 ± 0.04	2.17 ± 0.03	2.21 ± 0.14	2.26 ± 0.23
	10	2.13 ± 0.04	2.15 ± 0.03	2.23 ± 0.17	2.25 ± 0.14
9Ba	8	2.08 ± 0.07	2.22 ± 0.11	2.20 ± 0.13	2.33 ± 0.52
	4	2.08 ± 0.07	2.24 ± 0.15	2.57 ± 0.65	2.68 ± 0.52
	2	2.13 ± 0.07	2.35 ± 0.17	2.67 ± 0.69	2.88 ± 0.63
9Bb	12	2.21 ± 0.15	2.20 ± 0.11	2.59 ± 0.45	2.86 ± 0.92
	5	2.15 ± 0.13	2.32 ± 0.15	2.56 ± 0.44	3.09 ± 0.72
	2.25	2.15 ± 0.08	2.18 ± 0.09	2.75 ± 0.87	2.75 ± 0.69
	1.25	2.17 ± 0.18	2.24 ± 0.12	2.47 ± 0.41	2.61 ± 0.56
9Ca	12	2.14 ± 0.04	2.11 ± 0.05	2.07 ± 0.06	*d*

[a] Each dose tested consisted of eight animals; the untreated controls represent the average of four experiments for a total of 48 animals. [b] Paw swelling is expressed as the mean ± standard deviation of hind paw dimensions (mm) for both limbs of all animals in each group. Hind paw dimensions average 2.13 ± 0.09 mm in the absence of collagen challenge; arthritic animals typically exhibit hind paw dimensions greater than 2.60 ± 0.50 mm. [c] One death was observed in this group. A dose of 9 mg/kg was adopted as a standard for MTX; lower doses were not as uniformly effective. [d] Three animals died.

tions raise the possibility that the mode of action of methotrexate and the methotrexate analogues may be through selective inhibition of cell proliferation. Although our studies do not directly address the mechanism of action of methotrexate, the differential response of the disease to unique structures of the methotrexate analogues may provide insights into mechanisms underlying drug effects on disease progression.[29,30]

This study indicates that modification of the MTX structure can provide compounds that are as effective if not more so than MTX against rheumatoid arthritis. It further suggests that an antiproliferative effect is paramount in the mechanism of action. Since the target cell(s) has not been identified, we cannot yet conduct a detailed structure−activity study that would allow determination of specific cytotoxicity and influx/efflux parameters vs sensitive host cells as we have conducted for cancer cell types. However, acute and subchronic toxicity studies in rodents for **9Ab** do suggest a modest therapeutic advantage for this compound over MTX. At this stage we can conclude that the thiophene moiety offers potential as a side chain substituent for development of additional 10-deazaaminopterin-based agents.

Experimental Section

Elemental analyses were obtained from Galbraith Laboratories, Knoxville, TN, and Atlantic Microlab, Norcross, GA. Values were within 0.4% of theory unless noted in parentheses. Mass spectra were run on an LKB 9000 GC−MS spectrometer or a Ribermag R10-10C MS system. Ultraviolet spectra were taken on a Perkin-Elmer 552 or Perkin-Elmer-Coleman 575 instrument. ^{1}H-NMR spectra were recorded on a Varian Gemini 300 spectrometer. Melting points were determined on a Thomas-Hoover Unimelt apparatus. HPLC analyses were conducted on a Spectra Physics 8100 XR unit using a Novapak C18 column with elution by 25% MeOH in 0.1 M NaH_2PO_4 buffer, pH 6.5, with detection at 254 nm.

2-Carboxythiophene-5-acetic Acid Dimethyl Ester (2A). Freshly distilled diisopropylamine (24.6 g, 0.24 mol) in 250 mL of dry THF was cooled to 0 °C under argon and then treated dropwise with 98 mL (0.24 mol) of 2.5 M BuLi in hexane. After 1 h, the LDA solution was added dropwise with stirring to a −30 °C mixture of 15.0 g (0.11 mol) of 5-methylthiophene-2-carboxylic acid (**1A**) in 300 mL of dry THF. The temperature of the resulting red solution was allowed to rise to 0 °C and was so maintained for another 2 h. Carbon dioxide was bubbled through the solution to produce a yellow precipitate. The mixture was stirred at ambient temperature for 2 h and filtered. The yellow filter cake was suspended in 300 mL of MeOH, and the mixture was cooled to 0 °C and treated with 100 mL of MeOH saturated with dry HCl. The mixture was stirred at room temperature for 72 h and concentrated *in vacuo*, and the residue was partitioned between Et_2O (500 mL) and 250 mL of saturated $NaHCO_3$. The Et_2O extract was washed with H_2O (3 × 250 mL), dried over $MgSO_4$, and evaporated to leave a dark oil (15 g). Chromatography on flash silica gel (EtOAc−hexane, 1:19) gave 11.4 g of the product (51%) as a white, waxy solid: NMR ($CDCl_3$) δ 7.61 (d, 1H, 3-H), 6.90 (d, 1H, 4-H), 3.87 (m, 5H, $ArCOOCH_3$ + CH_2), 3.82 (s, 3H, CH_2COOCH_3). Anal. ($C_9H_{10}O_4S$) C, H, N.

3-Carboxypyridine-6-acetic acid dimethyl ester (2B): This diester was similarly prepared from 6-methylnicotinic acid (**1B**) as a yellow solid; mp 56−57 °C; NMR ($CDCl_3$) δ 9.10 (m, 1H, 6-H), 8.21 (m, 1H, 4-H), 7.33 (m, 1H, 3-H), 3.84 (m, 8H, CH_2COOCH_3, $ArCOOCH_3$). Anal. ($C_{10}H_{11}NO_4$) C, H, N.

(2-Carboxy-5-pyridyl)acetic acid dimethyl ester (2C): prepared in a manner similar to **2A** from 5-methylpicolinic acid[31] giving the product as an amber oil in 49% yield; NMR ($CDCl_3$) δ 8.63 (d, 1H, C_3-H), 8.15 (d, 1H, C_6-H), 7.81 (m, 1H, C_4-H), 4.02 (s, 3H $ArCOOCH_3$), 3.75 (s, 5H, CH_2COOCH_3).

(2-Carbomethoxy-5-pyridyl)acetic Acid Benzhydryl Ester (4C). A solution of KOH (1.39 g, 24.8 mmol) in 90% MeOH (100 mL) was treated with a solution of **2C** (5.18 g, 24.8 mmol) in MeOH (14 mL). After 2 h, the solution was adjusted to pH 6.5 by addition of 2 N HCl. The solution was concentrated *in vacuo* to give a tan solid that was a mixture of both monoesters, the dicarboxylic acid and the starting diester. HPLC indicated the desired monoester **3C** to represent 57% of the mixture.

The mixture in $CHCl_3$ (100 mL) was cooled to 0° and treated dropwise with a solution of diphenyldiazomethane (5.27 g, 27.2 mmol) in $CHCl_3$ (50 mL). The resulting purple mixture was stirred at ambient temperature for 24 h. The solution was washed with 25 mL of saturated $NaHCO_3$ and 25 mL of H_2O. The organic layer was dried over $MgSO_4$ and concentrated to a purple syrup. Crystallization from pentane gave the product as a white solid: 1.86 g (21% yield from **2C**); mp 90−92 °C NMR ($CDCl_3$) δ 8.68 (m, 1H, C_3-H), 8.10 (d, 1H, C_6-H), 7.75 (m, 1H, C_4-H), 7.30 (m, 10H, 2 × C_6H_5), 6.90 (s, 1H, OCH), 4.05 (s, 3H, OCH_3), 3.81 (s, 2H, CH_2). Anal. ($C_{22}H_{19}NO_4 \cdot 0.25H_2O$) C, H, N.

α-Ethyl-2-carboxythiophene-5-acetic Acid Dimethyl Ester (3A). A suspension of 0.59 g of 50% sodium hydride in oil (12.2 mmol of sodium hydride) in 20 mL of dry dimethylformamide was cooled to 0 °C. A solution of **2A** (2.60 g, 12.2 mmol) in 20 mL of dry dimethylformamide was added, and the reaction mixture was stirred for an additional 1 h at 0 °C. The reaction mixture was cooled to −30 °C, treated dropwise with a solution of ethyl iodide (1.9 g, 12.2 mmol) in dry dimethylformamide (10 mL), and then stirred for 2.5 h at 20 °C. The solution was neutralized (pH 8) by adding solid carbon dioxide, and then concentrated under high vacuum. The residue was digested in ether (250 mL) and filtered. The filtrate was washed with 100 mL of H_2O and then 50 mL of 10% sodium bisulfite and 100 mL of H_2O. The organic layer was dried over $MgSO_4$ and evaporated *in vacuo*. The residue was chromatographed on flash silica gel (EtOAc−hexane, 1:19) to yield the product as a clear, colorless oil: 1.7 g (58%); TLC (10% ethyl acetate in hexane on silica gel plate) R_f 0.35; NMR ($CDCl_3$) δ 7.59 (d, 1H, Ar 3-H), 7.20 (d, 1H, Ar 4-H), 3.81 (m, 7H, 2 × OCH_3 + Ar*CH*), 2.06 (m, 2H, CH_2CH_3), 0.95 (t, 3H, CH_3). Anal. ($C_{11}H_{14}O_4S$) C, H.

α-Ethyl(3-carboxy-6-pyridyl)acetic acid dimethyl ester (3B): similarly prepared from 3-carboxypyridine-6-acetic acid dimethyl ester (**2B**) in 51% yield as a yellow oil; NMR ($CDCl_3$) δ 9.13 (m, 1H, 6-H), 8.26 (m, 1H, 4-H), 7.39 (m, 1H, 3-H), 3.83 (m, 7H, 2 × OCH_3 + α-CH), 2.10 (m, 2H, CH_2CH_3), 0.87 (t, 3H, CH_3CH_2). Anal. ($C_{12}H_{15}NO_4S$) C, H, N.

Methyl 5-[α-Carbomethoxy-β-(2,4-diamino-6-pteridinyl)ethyl]thiophene-2-carboxylate (5Aa). A suspension of 50% sodium hydride in oil (0.84 g, 17.5 mmol of sodium hydride) in 15 mL of dry dimethylformamide was cooled to 0 °C. A solution of the diester **2A** (3.73 g, 17.4 mmol) in 15 mL of dry dimethylformamide was added dropwise. The resulting mixture was stirred at 0 °C for 1 h and then cooled to −30 °C and treated with a solution of 2,4-diamino-6-(bromomethyl)-pteridine hydrobromide (16.1 mmol) in 40 mL of dry dimethylformamide. The resulting mixture was stirred for 2.5 h while rising to room temperature and then neutralized (pH 7.5) by adding solid carbon dioxide. The mixture was concentrated under high vacuum, and the residue was washed with ether and then water and dried under high vacuum to give the product as a yellow solid (1.98 g, 88%): MS 389 (M + H); NMR (DMSO-d_6) δ 8.58 (s, 1H, C_7-H), 7.60 (m, 3H, C_3'-H + NH_2), 7.12 (d, 1H, C_4'-H), 6.61 (br s, 2H, NH_2), 4.9 (t, 1H, C_{10}-H), 3.75 (s, 3H, C_2'-$COOCH_3$), 3.63 (m, 5H, C_{10}-$COOCH_3$ + C_9-H_2). Anal. ($C_{16}H_{16}N_6O_4S \cdot 0.5H_2O$) C, H, N.

Methyl 5-[α-carbomethoxy-α-[(2,4-diamino-6-pteridinyl)methyl]propyl]thiophene-2-carboxylate (5Ab): similarly prepared from **3A** in 85% yield as a yellow solid; NMR (DMSO-d_6) δ 8.35 (s, 1H, C_7-H), 7.78 (br s, 1H, NH), 7.65 (d, 1H, C_3'-H), 7.17 (d, 1H, C_4'-H), 6.65 (br s, 2H, NH_2), 6.52 (br s, 1H, NH), 3.77 (s, $ArCOOCH_3$), 3.68 (s, $CCOOCH_3$), 2.06 (m, 2H, CH_2CH_3), 0.76 (t, 3H, CH_3CH_2); MS *m/e* 416 (M + H). Anal. ($C_{18}H_{20}N_6O_4S$) C, H, N.

Methyl 6-[α-carbomethoxy-β-(2,4-diamino-6-pteridinyl)-ethyl]pyridine-3-carboxylate (5Ba): similarly prepared from **2B** in 99% yield as a yellow solid; NMR (DMSO-d_6) δ 9.00 (m, 1H, C_7-H), 8.53 (s, 1H, C_2'-H), 8.22 (m, 1H, C_4'-H), 7.55 (m, 3H, C_5'-H + NH_2), 6.55 (br s, 2H, NH_2), 4.92 (t, 1H,

C_{10}-H), 3.85 (s, 3H, $COOCH_3$), 3.75 (m, 4H C_9H + $COOCH_3$), 3.38 (m, C_9H), Anal. ($C_{17}H_{17}N_7O_4 \cdot 1.7H_2O$) C, H (0.75), N.

Methyl 6-[α-carbomethoxy-α-[(2,4-diamino-6-pteridinyl)methyl]propyl]pyridine-3-carboxylate (5Bb): similarly obtained from **3B** in 78% yield as a yellow solid; MS *m/e* 412 (M + H); NMR (DMSO-d_6) δ 9.04 (s, 1H, C_7-H), 8.23 (m, 2H, pyr 2′-H + pyr 4′-H), 7.45 (d, 1H, pyr 5′-H), 6.62 (br s, 2H, NH_2), 3.87 (s, 3H, $ArCOOCH_3$), 3.62 (m, 5H, C_{10}-$COOCH_3$ + C_9-H_2); 2.01 (m, 2H, CH_2CH_3), 0.80 (t, 3H, CH_3CH_2). Anal. ($C_{19}H_{21}N_7O_4 \cdot 1.5H_2O$) C, H, N.

Methyl 5-[α-[(benzhydryloxy)carbonyl]-β-(2,4-diamino-6-pteridinyl)ethyl]pyridine-2-carboxylate (5C): prepared in a similar manner from **4C** in 75% yield as a yellow powder; NMR ($CDCl_3$) δ 8.80 (m, 1H, C_7-H), 8.62 (s, 1H, C_3′-H), 8.10 (d, 1H, C_6′-H), 7.84 (m, 1H, C_4′-H), 7.20 (m, 12H, 2 × C_6H_5 + NH_2), 6.80 (s, 1H, O*CH*), 5.20 (br s, 2H, NH_2), 4.55 (m, 1H, C_{10}-H), 4.02 (s, 3H, OCH_3), 3.85 (m, 1H, C_9-H), 3.30 (m, 1H, C_9-H).

5-[α-Carboxy-β-(2,4-diaminopteridinyl)ethyl]thiophene-2-carboxylic Acid (6Aa). A solution of the diester **5Aa** (1.96 g, 5.05 mmol) in 30 mL of 2-methoxyethanol and 30 mL of 2.5 N NaOH was stirred for 1.5 h. The mixture was filtered, and the filtrate was neutralized (pH 7) with HOAc and concentrated under high vacuum. The residue was suspended in water (30 mL) and adjusted with HOAc to pH 5 to produce a precipitate. Filtration gave a tan solid that was digested in 95% EtOH and filtered to give a tan solid that was washed with Et_2O and dried *in vacuo*, yielding 1.31 g (77%) of product: HPLC showed 92.2% purity; NMR (DMSO-d_6) δ 8.51 (s, 1H, C_7-H), 7.55 (br s, 2H, NH_2), 7.17 (d, 1H, 3′-H), 6.81 (d, 1H, 4′-H), 6.55 (br s, 2H, NH_2), 4.40 (t, 1H, C_{10}-H), 3.15 (m, 2H, C_9-H_2).

5-[β-(2,4-Diamino-6-pteridinyl)ethyl]thiophene-2-carboxylic Acid (7Aa). A solution of the dicarboxylic acid **6Aa** (1.31 g, 3.64 mmol) in argon-purged DMSO was placed in a 135 °C oil bath for 45 min. The solution was then concentrated under high vacuum to a residue that was digested in Et_2O (50 mL). Filtration yielded a brown solid that was washed with ether and dried *in vacuo* to give 1.31 g of crude product. The material was suspended in water (75 mL) and treated dropwise with 1.5 N NH_4OH to pH 12. Insoluble material was removed by filtration and the filtrate adjusted to pH 5 with HOAc to give a precipitate. Filtration gave a brown solid that was washed with H_2O and dried *in vacuo*, yielding 0.97 g of product (84%): UV (0.1 N NaOH) 257 nm (ϵ 25 305), 372 (6491). Anal. ($C_{13}H_{12}N_6O_2S \cdot H_2O$) C, H, N.

5-[α-[(2,4-Diamino-6-pteridinyl)methyl]propyl]-2-thenoic Acid (7Ab). The diester **5Ab** was saponified via the above procedure used to prepare **6Aa** to yield (58%) **6Ab** as a cream-colored solid: HPLC 97% purity. The material was immediately decarboxylated by heating a solution in DMSO at 125° for 30 min to afford the α-ethylpteroate analogue **7Ab** in 70% yield: UV (0.1 N NaOH) 256 nm (ϵ 28 546), 372 (7300); MS (DCl−NH_3) *m/e* 561 (TMS_3) corresponds to 345 (M + 1). Anal. ($C_{15}H_{16}N_6O_2S \cdot 0.6H_2O$) C, H, N.

6-[β-(2,4-Diamino-6-pteridinyl)ethyl]pyridine-3-carboxylic Acid (7Ba). Saponification of the diester **5Ba** afforded the diacid **6Ba** in 97% yield; HPLC showed 95.3% purity. The material was directly decarboxylated by heating a solution in DMSO at 110 °C for 25 min to give **7Ba** in 94% yield as a yellow solid: MS *m/e* 527 (TMS_3) corresponds to 311 (M + 1). Anal. ($C_{14}H_{13}N_7O_2 \cdot 2H_2O$) C (0.6), H, N.

6-[α-[(2,4-Diamino-6-pteridinyl)methyl]propyl]pyridine-3-carboxylic Acid (7Bb). Similar hydrolysis of the diester **5Bb** gave the diacid **6Bb** in 27% yield as a tan solid. Decarboxylation was effected by allowing a solution in DMF to stand at room temperature for 20 min to afford **7Bb** in 99% yield; HPLC showed 90% purity.

Methyl 5-[β-(2,4-Diamino-6-pteridinyl)ethyl]pyridine-2-carboxylate (7C). A mixture of the diester **5C** (1.29 g, 2.4 mmol) in dichloromethane (67 mL) was treated with 99% trifluoroacetic acid (33 mL). The yellow solution was kept at room temperature for 50 min and then concentrated at room temperature under high vacuum. The residue was washed repeatedly with ether and then dried *in vacuo* giving a bright yellow solid. This was suspended in water and neutralized to pH 8 with 1.5 N ammonium hydroxide. The mixture was concentrated under high vacuum giving a yellow solid, 0.99 g. HPLC showed the conversion to **6C**.

A solution of the monocarboxylic acid **6C** (0.99 g, crude) in 40 mL of dimethyl sulfoxide was stirred at 130° for 30 min. HPLC showed disappearance of the starting carboxylic acid **6C** (retention time 4.4 min) and the desired monoester to be present (retention time 15.2 min). The solution was concentrated under high vacuum, and the residue was washed with ether and water. The orange solid was collected and dried *in vacuo* at room temperature to afford 505 mg (64%): NMR ($CDCl_3$) δ 8.60 (m, 2H, C_7-H, 6′-H), 8.10 (d, 1H, 3′-H), 7.85 (d, 1H, 5′-H), 7.20 (m, 3H, NH_2), 4.00 (s, 3H, OCH_3), 3.35 (s, 4H, CH_2CH_2); MS *m/e* 325 (M + H).

5-[β-(2,4-Diamino-6-pteridinyl)ethyl]pyridine-2-carboxylic Acid (7Ca). A mixture of the ester **7C** (0.49 g, 1.5 mmol) in 2-methoxyethanol (5 mL) was treated with water (5 mL) and then 10% NaOH (2.5 mL). HPLC analysis indicated saponification was complete after the resulting red solution had been stirred for 45 min. The solution was neutralized (pH 7.5) with 2 N HCl and concentrated under high vacuum. The resulting residue was treated with water and stirred. Filtration gave 0.27 g of product as an orange solid (57%): HPLC showed 96% purity; MS *m/e* 527 (TMS_3). Anal. ($C_{14}H_{13}N_7O_2 \cdot 0.8HCl$) C, H, N.

Diethyl *N*-5-[β-(2,4-Diamino-6-pteridinyl)ethyl]-2-thenoyl]-L-glutamate (8Aa). A solution of the carboxylic acid **7Aa** (0.7 g, 2.2 mmol) in dry DMF (40 mL) was treated with triethylamine (2.1 g, 21.0 mmol) and stirred at room temperature for 1.25 h. Isobutyl chloroformate (0.63 g, 4.6 mmol) was added, and the mixture was stirred at room temperature for 2 h. Isobutyl chloroformate (0.32 g, 2.3 mmol) was again added, and the mixture was stirred for 1 h followed by addition of LL-glutamic acid diethyl ester hydrochloride (0.55 g, 2.3 mmol); stirring was continued for another 1 h. The process was repeated again, and the mixture was stirred at room temperature overnight. Concentration under high vacuum gave a dark residue that was washed repeatedly with Et_2O. The residue was then washed with dilute NH_4OH, and then H_2O. The resultant orange solid was dried *in vacuo*. Chromatography on flash silica gel (2.5% MeOH in $CHCl_3$) gave the product as a yellow powder: 0.32 g (32%); mp 206−208 °C; NMR (DMSO-d_6 + $CDCl_3$) δ 8.5 (s, 1H, C_7-H), 8.31 (d, 1H, *NH*C), 7.6 (d, 1H, 3′-H), 6.80 (d, 1H, 4′-H), 6.32 (br s, 2H, NH_2), 4.54 (m, 1H, *CH*NH), 4.18 (m, 4H, 2 × OCH_2), 3.28 (m, C_9-H_2); 2.42 (t, 2H, Glu C_4-H_2); 2.13 (m, 2H, Glu C_3-H_2); 1.28 (m, 6H, 2 × CH_3CH_2); MS *m/e* 502 (M + H). Anal. ($C_{22}H_{27}N_7O_5S \cdot H_2O$) C, H, N.

The other L-glutamate diesters were similarly prepared from the corresponding "pteroic acid" intermediates.

Diethyl *N*-[5-[α-[(2,4-diamino-6-pteridinyl)methyl]propyl]-2-thenoyl]-L-glutamate (8Ab): 20% yield from **7Ab**, yellow foam; NMR ($CDCl_3$) δ 0.90 (t, 3H, C_{10}-CH_2-CH_3), 1.30 (m, 6H, 2 × OCH_2CH_3), 2.17 (m, 2H, Glu C_3-H_2); 2.47 (m, 2H, Glu C_4-H_2), 3.20 (m, 3H, C_9-H_2 + C_{10}-H), 4.16 (m, 4H, 2 × OCH_2), 4.75 (m, 1H, *CH*NH), 5.45 (br s, NH), 6.55 (m, 1H, C_4′-H), 6.95 (m, 1H, *NH*CH), 7.30 (d, 1H, C_3′-H), 8.41 (d, 1H, C_7H). Anal. ($C_{24}H_{31}N_7O_5S \cdot 0.7H_2O$) C, H, N.

Diethyl *N*-[6-[β-(2,4-diamino-6-pteridinyl)ethyl]nicotinoyl]-L-glutamate (8Ba): 50% yield from **7Ba**, yellow crystals from EtOH; MS *m/e* 497 (M + H); NMR (DMSO-d_6) δ 8.90 (d, 1H, NHCO), 8.87 (d, 1H, pyr 6′-H), 8.61 (s, 1H, C_7-H), 8.10 (m, 1H, pyr 4′-H), 7.70 (br d, 1H, NH), 7.42 (d, 1H, pyr 3′-H), 6.65 (br s, 2H, NH_2), 4.40 (m, 1H, *CH*N), 4.05 (m, 4H, 2 × OCH_2), 3.30 (CH_2CH_2 + H_2O), 2.45 (t, 2H, CH_2CO_2), 2.05 (m, 2H, CH_2CH), 1.70 (t, 6H, 2 × CH_3). Anal. ($C_{23}H_{28}N_8O_5 \cdot H_2O$) C, H, N.

Diethyl *N*-[6-[α-[(2,4-diamino-6-pteridinyl)methyl]propyl]nicotinoyl]-L-glutamate (8Bb): 48% from **7Bb**, yellow foam; MS *m/e* 525 (M + H); NMR ($CDCl_3$) δ 9.01 (br s, 1H, pyr 6′-H), 8.45 (br s, 1H, 7-H), 7.97 (d, 1H, pyr 4′-H), 7.35 (br s, 2H, NH_2), 7.08 (d, 1H, pyr 3′-H), 5.38 (br s, 2H, NH_2), 4.75 (m, 1H, *CH*N), 4.19 (m, 4H, 2 × OCH_2), 3.32 (m, 3H, C_9-H_2 + C_{10}-H), 2.50 (m, 2H, C_{10}-CH_2-CH_3), 2.23 (m, 4H, Glu C_4-H_2 + Glu C_3-H_2); 1.26 (m, 6H, 2 × OCH_2CH_3), 0.83 (t, 3H, C_{10}-CH_2CH_3).

Diethyl *N*-[5-[β-(2,4-diamino-6-pteridinyl)ethyl]picolinoyl]-L-glutamate (8Ca): 18% from **7Ca**, yellow foam; MS *m/e* 497 (M + H); NMR ($CDCl_3$) δ 8.60 (d, 1H, C_7-H), 8.55 (d, 1H, NH), 8.43 (d, 1H, C_5′-H), 8.06 (d, 1H, C_2-H), 7.70 (m, 1H, C_6′-H), 4.80 (m, 1H, *CH*NH), 4.20 (m, 4H, 2 × OCH_2), 2.30 (m,

4H, Glu CH_2CH_2), 1.30 (m, 6H, 2 × OCH_2CH_3). Anal. ($C_{23}H_{28}N_8O_5$) C, H, N.

***N*-[5-[*β*-(2,4-Diamino-6-pteridinyl)ethyl]-2-thenoyl]-L-glutamic Acid (9Aa).** A mixture of the diester **8Aa** (0.26 g, 0.5 mmol) in 2-methoxyethanol (5 mL) was treated with water (5 mL) and 10% NaOH (5 mL). The mixture was stirred for 1 h and then adjusted to pH 5.5 with 2 N HCl and concentrated under high vacuum. The residue was digested in H_2O (5 mL), and the mixture was filtered. The resulting solid was washed with H_2O and dried *in vacuo* to afford 0.19 g of product (82%): HPLC showed 96.4% purity; UV (0.1 N NaOH) 258 nm (*ϵ* 28 310), 372 (6737); NMR (DMSO-d_6) *δ* 8.67 (s, 1H, C_7-H), 8.50 (d, 1H, *NH*CH), 8.00 (br s, 2H, NH_2), 7.65 (d, 1H, 3′-H), 6.90 (br s, 3H, 4′-H + NH_2), 4.30 (m, 1H, *CH*NH), 3.42 (m, C_9-H_2 + C_{10}-H_2); 2.35 (t, 2H, Glu C_4-H_2); 1.95 (m, 2H, Glu C_3-H_2); MS (DCl−NH_3) *m*/*e* 734 (TMS_4) (M + H). Anal. ($C_{18}H_{19}N_7O_5S$·$2H_2O$) C, H, N.

The other aminopterin analogues were similarly obtained by saponification of the glutamate esters.

***N*-[5-[α-[(2,4-Diamino-6-pteridinyl)methyl]propyl]-2-thenoyl]-L-glutamic acid (9Ab):** 57% yield from **8Ab**, yellow solid; HPLC 97% purity; UV (0.1 N NaOH) 256 nm (*ϵ* 28 139), 371 (6810); MS (DCl−NH_3) *m*/*e* 762 for TMS_4 (M + H). Anal. ($C_{20}H_{23}N_7O_5S$·0.75 HCl) C, H, N.

***N*-[6-[*β*-(2,4-Diamino-6-pteridinyl)ethyl]nicotinoyl]-L-glutamic acid (3′-aza-10-deazaaminopterin) (9Ba):** 71% yield from **8Ba**, yellow powder; HPLC 95% purity; UV (0.1 N NaOH) 258 nm (*ϵ* 25 000), 275 sh (13 900), 371 (6600); MS (DCl−NH_3) *m*/*e* 729 (TMS_4) (M + H). Anal. ($C_{19}H_{20}N_8O_5$·$2.25H_2O$) C, H (0.5), N.

***N*-[6-[α-[(2,4-Diamino-6-pteridinyl)methyl]propyl]nicotinoyl]-L-glutamic acid (3′-aza-10-ethyl-10-deazaaminopterin) (9Bb):** 39% from **8Bb**, yellow powder; HPLC 98.9% purity; UV (0.1 N NaOH) 256 nm (*ϵ* 25 246), 367 (6562). Anal. ($C_{21}H_{24}N_8O_5$·1.4 H_2O) C, H, N.

***N*-[5-[*β*-(2,4-Diamino-6-pteridinyl)ethyl]picolinoyl]-L-glutamic acid (2′-aza-10-deazaaminopterin) (9Ca):** 58% yield from **8Ca**, yellow solid; HPLC 99.3% purity; UV (0.1 N NaOH) 257 nm (*ϵ* 24 000), 371 (6070); MS (DCl−NH_3) *m*/*e* 729 (TMS_4) (M + H). Anal. ($C_{19}H_{20}N_8O_5$·$0.25H_2O$) C, H, N.

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