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Communications to the Editor

The 2-Desamino and 2-Desamino-2-methyl Analogues of Aminopterin Do Not Inhibit Dihydrofolate Reductase but Are Potently Toxic to Tumor Cells in Culture[1]

Sir:

The 2-amino group in classical folic acid analogues such as methotrexate (MTX, **1**) and aminopterin (AMT, **2**) is conventionally thought to be an essential feature of the molecule where biological activity is concerned.[2] Support for this view has come, historically, from the fact that analogues in which the 2-amino group was replaced by *N*,*N*-dimethylamino[3] or methylthio[4] groups were essentially devoid of activity. More recently, much stronger support has been provided by X-ray crystallographic studies[5-7] showing that the 2-amino group of MTX is involved in hydrogen bonding to a highly conserved aspartic or glutamic acid residue in the active site of the target enzyme dihydrofolate reductase (DHFR), as well as to a molecule of water which is hydrogen bonded in turn to a threonine residue of the enzyme. Replacement of the aspartic acid residue in *E. coli* DHFR with serine by site-directed mutagenesis has been shown to produce a 3000-fold increase in the K_D for MTX binding as measured by equilibrium dialysis.[8,9] This substantial change corresponds to a decrease of 4.4 kcal mol^{-1} in the binding energy. In another study,[10] replacement of the threonine residue by valine, whose side chain cannot participate in hydrogen bonding, was found to produce a 25-fold increase in the K_D for MTX, a smaller effect nonetheless corresponding to a free energy difference of almost 2 kcal mol^{-1}. A further important role for the 2-amino group is to provide resonance delocalization of the positive charge when N^1 is protonated during the binding of MTX to DHFR.[11] Thus, deletion of the 2-amino group would seem a very unpromising approach to the design of DHFR inhibitors.

The first intimation that a 2-amino group may not be an absolute requirement for inhibition of folate enzymes other than DHFR came recently from the work of Jones and co-workers,[12] who showed that the 2-amino group in the potent thymidylate synthase (TS) inhibitor 10-propargyl-5,8-dideazafolic acid (CB3717) could be replaced by hydrogen with only an 8-fold loss of TS inhibition and a 10-fold increase in potency against L1210 murine leukemia cells in culture. Replacement of the 2-amino group with methyl was subsequently reported[13] to give a 2-fold decrease in anti-TS activity with a 40-fold increase in cytotoxicity. Interestingly, these compounds were also found to be folylpolyglutamate synthetase (FPGS) substrates,[14] leading to the conclusion that the 2-amino group is not absolutely required for FPGS binding, just as it is not essential for binding to TS. According to another recent report,[15] the 2-desamino analogue of 5,8-dideazaisofolic acid (IAHQ) is much less active than the parent 2-amino-4-oxo compound as a TS inhibitor, but is none-

(1) Paper 36 in this series; for previous paper, see: Rosowsky, A.; Forsch, R. A.; Moran, R. G. *J. Med. Chem.* **1989**, *32*, 709.
(2) For a general review of strategies used in the design of classical antifolates, see: Piper, J. R.; Montgomery, J. A. In *Folate antagonists as Therapeutic Agents*; Sirotnak, F. M., Burchall, J. J., Ensminger, W. D., Montgomery, J. A., Ed.; Academic Press: Orlando, 1984, pp 219–260.
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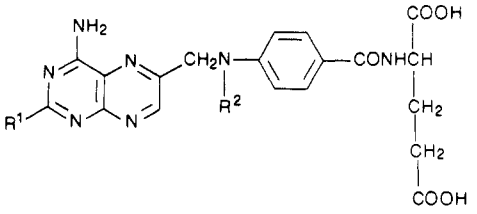
theless several times more potent against L1210 cells in culture.

The biological activity of the desamino analogues of CB3717 and IAHQ prompted us to synthesize similar desamino analogues of the classical DHFR inhibitor **2** with a view to determining (a) whether replacement of the amino group by hydrogen or methyl would decrease DHFR inhibition and (b) whether these structural changes would abolish inhibition of tumor cell growth. In the present communication we report the synthesis and preliminary biological evaluation of the desamino analogues **3** and **4** of AMT.

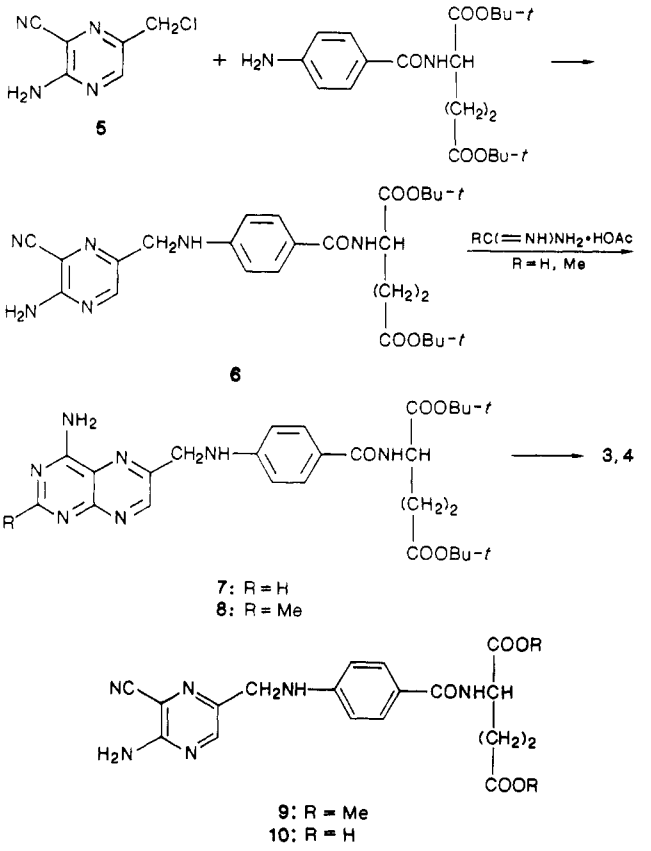


1: $R^1 = NH_2$, $R^2 = Me$
2: $R^1 = NH_2$, $R^2 = H$
3: $R^1 = R^2 = H$
4: $R^1 = Me$, $R^2 = H$

Our synthesis of **3** and **4** is outlined in Scheme I, and is an adaptation of an approach originally described by Taylor[16] as an unambiguous route to 6-substituted 2,4-diaminopteridines. 2-Amino-5-(chloromethyl)-3-cyanopyrazine (**5**)[17,18] was condensed with 1 molar equiv of di-*tert*-butyl *N*-(4-aminobenzoyl)-L-glutamate[19] in DMF solution in the presence of *i*-Pr_2EtN to obtain a mixture of the *N*-alkyl and *N,N*-dialkyl derivatives. After separation of this mixture by column chromatography on neutral alumina, pure **6** was isolated in 59% yield. Ring closure of the amino nitrile **6** was then achieved in 59% yield by treatment with a 4-fold molar excess of formamidine acetate[20] in refluxing 2-ethoxyethanol. Acidolysis of the resulting diester **7** was found to proceed smoothly at room temperature in 3 h in a 2:1 mixture of CH_2Cl_2 and CF_3COOH, giving **3** ("2-desaminoAMT") in 80–90% yield as a monohydrate. The UV absorption spectrum of **3** [λ_{max} (pH 7.4) 246 (ϵ 19 200), 285 (21 200), 333 (7240) nm; λ_{max} (0.1 N HCl) 293 nm (ϵ 16 700)] exhibited the expected hypsochromic shift for a 4-aminopteridine in comparison with a 2,4-diaminopteridine.[21] The NMR spectrum, in D_2O containing enough K_2CO_3 to dissolve the sample, contained two downfield singlets at δ 8.85 and 8.33 corresponding to hydrogens at position 7 and 2, respectively. Replacement of formamidine acetate with acetamidine acetate[20] in the foregoing sequence afforded the diester **8** (54% yield), which on treatment with 2:1 CH_2Cl_2–CF_3COOH was converted to **4** in 88% yield. The UV spectrum of **4** [λ_{max} (pH 7.4) 247 (ϵ 22 100), 285 (22 700), 337 (7670); λ_{max} (0.1 N HCl) 219 (ϵ 20 900), 293 (19 700)] was consistent with its assigned structure as a 4-amino-2-methylpteridine. The NMR spectrum of the diester **8**, in $CDCl_3$ solution, showed the 2-methyl group as a singlet at δ 2.68.

Two alternative routes to **3** were initially explored and

Scheme I



9: R = Me
10: R = H

abandoned because they were less satisfactory than the one described above. In one approach, **5** was condensed with dimethyl *N*-(4-aminobenzoyl)-L-glutamate instead of with the di-*tert*-butyl ester. The expected dimethyl ester **9** was obtained in 52% yield, but unfortunately when **9** was allowed to react with formamidine acetate in 2-ethoxyethanol, a mixture of transesterified products was formed which made purification difficult. Furthermore, when the crude product mixture was treated with 0.1 N NaOH to hydrolyze the ester groups, there was a change in UV spectral absorption indicating probable cleavage of the pyrimidine ring. While ester cleavage could be effected more satisfactorily with $Ba(OH)_2$, the yield of **3** was still only 33%. An attempt was made then to carry out the formamidine acetate reaction with diacid **10**, which could be obtained either from **5** and *N*-(4-aminobenzoyl)-L-glutamic acid (31% yield) or from **6** by acidolysis with CF_3COOH (55% yield). While it appeared that **3** was formed from **10** in small amount, there was again a large number of byproducts which made purification difficult. It was clear from these experiments that annulation would best be carried out with the carboxyl groups blocked and that the best blocking group would be a *tert*-butyl ester, which would be more stable than a methyl ester to transesterification and would be removable at the end with acid as opposed to alkali.

We had anticipated that removal of the electron-donating group from the 2-position would decrease the stability of the pteridine ring not only to alkaline but also to acid conditions. This was borne out by the finding that, in contrast to our previous experience with di-*tert*-butyl esters of analogues containing an intact 2,4-diaminopteridine ring,[22] treatment of **6** with neat CF_3COOH

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resulted in extensive decomposition. Fortunately, when the reaction was conducted in a 2:1 mixture of CH_2Cl_2 and CF_3COOH, the 4-aminopyrimidine moiety remained intact and the *tert*-butyl esters were removed in quantitative yield.

Solutions of **3** and **4** in 0.1 N NaOH appeared to undergo rapid chemical change, as evidenced by the appearance of new peaks on HPLC analysis. However, solutions in pH 7.4 phosphate buffer were stable at room temperature for at least 24 h, and only after some time ($t_{1/2} \approx 3$ days) began to show changes suggestive of pyrimidine ring cleavage. This tendency toward slow ring opening at physiologic pH could be a useful property in that it might minimize long-term accumulation of these toxic compounds (or their presumably more toxic polyglutamates) in liver and kidney.

A potential problem that was considered in connection with our synthetic scheme was whether the use of formamidine or acetamidine at the relatively high temperature of refluxing 2-ethoxyethanol might cause racemization of the esterified amino acid side chain, resulting in a D,L mixture whose biological activity would be compromised. This problem has been recognized previously when annulation is effected with guanidine in refluxing ethanol,[23] and the enantiomeric purity of MTX is known to contribute to antitumor activity.[24,25] Evidence that compounds **3** and **4** were, in fact, enantiomerically pure was obtained by treatment with the enzyme carboxypeptidase G_1,[26] which cleaves L-MTX to 4-amino-4-deoxy-N^{10}-methylpteroic acid under conditions that leave D-MTX unaffected.[25] Brief incubation of the 2-desamino compounds with carboxypeptidase G_1 as previously described[27] and analysis of the course of reaction by HPLC showed rapid disappearance of all but a few percent of the starting material, as did incubation of commercial MTX. By contrast, D-MTX generated previously by treatment of commercial MTX with the enzyme remained unchanged. We conclude from these results that our desamino analogues are essentially pure L enantiomers. Use of carboxypeptidase G_1 appears to be a convenient method for the determination of enantiomeric purity in synthetic antifolates containing a glutamate side chain. The fact that annulation with formamidine and acetmidine acetate in refluxing 2-ethoxyethanol did not cause significant racemization validated our choice of the synthetic plan in Scheme I. However, it is clear that an alternative route to **3** and **4** involving condensation of di-*tert*-butyl L-glutamate with a preformed pteroic acid should also be possible.

As expected, removal of the 2-amino group resulted in a decrease in activity against purified DHFR from human leukemic lymphoblasts (WI-L2 cells)[28] of 1000-fold or more relative to MTX (Table I). To our surprise, however, growth-inhibitory activity against WI-L2 cells was decreased only 6-fold in the case of 2-desaminoAMT and 2-fold in the case of 2-desamino-2-methylAMT. Qualitatively similar results were obtained with L1210 murine leukemia cells, though the decrease in potency was somewhat larger for each compound. Thus, even though

Table I. Lack of Correlation between the Effects of 2-Desaminoaminopterin (**3**) and 2-Desamino-2-methylaminopterin (**4**) on DHFR Activity versus Cell Growth[a]

compd	DHFR IC_{50}, μM	IC_{50}, μM L1210	IC_{50}, μM WI-L2
MTX (**1**)	0.02	0.009	0.013
3	19	0.082	0.081
4	>50	0.042	0.028

[a] DHFR was isolated from MTX-resistant WI-L2 cells and purified by affinity chromatography as previously described.[28]

Table II. Relative Activity of AMT (**2**), 2-DesaminoAMT (**3**), and 2-Desamino-2-methylAMT (**4**) as Substrates for Mouse Liver FPGS

compd[a]	K_m, μM	V_{max}, relative[b]	V_{max}/K_m, relative[b]
2	22.8 ± 6.4	1.0	1.0
3	26.4 ± 1.4	0.87 ± 0.09	0.74 ± 0.09
4	31.7 ± 5.6	0.77 ± 0.01	0.60 ± 0.08

[a] FPGS was partially purified from mouse liver and kinetic constants were derived as previously described.[29] For compounds **2** and **3**, n (number of experiments on different days) = 3; for **4**, n = 2. [b] Relative to AMT in the same experiment.

Table III. Effect of Thymidine and Hypoxanthine on the Growth of L1210 Cells in the Presence of 2-Desaminoaminopterin (**3**) and 2-Desamino-2-methylaminopterin (**4**)[a]

compd	IC_{50}, μM standard medium	+ dThd	+ Hx	+ dThd and Hx
3	0.082	0.16	0.26	>100
4	0.042	0.13	0.16	>100

[a] Thymidine (dThd) and hypoxanthine (Hx) were added at concentrations of 10 and 100 μM, respectively.

3 and **4** were very poor DHFR inhibitors, they were still potently cytotoxic. Interestingly, these analogues also retained good substrate activity for FPGS. The K_m of **3** as a substrate for mouse liver FPGS (Table II) was found to be 26 μM, while that of **4** was 32 μM. Thus, the desamino analogues possessed FPGS substrate activity comparable to that of AMT, an antifol with very high substrate activity for this enzyme.[29]

Reversal experiments were performed to rule out any possibility that cell growth was inhibited as a result of something other than an antifolate effect. As shown in Table III, the addition of either 10 μM thymidine or 100 μM hypoxanthine alone was only partially effective in restoring normal growth of L1210 cells, while a combination of 10 μM thymidine and 100 μM hypoxanthine was fully protective. Similar results were obtained with WI-L2 cells (data not shown). This evidence supports the conclusion that **3** and **4** act primarily as antifolates, rather than as inhibitors of some biochemical process unrelated to the folate pathway. The exact locus at which **3** and **4** act in blocking cell growth is unknown at this time. However, on the basis of the excellent FPGS substrate activity these compounds possess in vitro, it is reasonable to assume that they form polyglutamates intracellularly and that the latter may be the species actually responsible for the observed in vitro antitumor activity.

Compounds **3** and **4** appear to be the first examples of a novel type of folate antagonist in which the 4-amino group of the classical antifolate structure is preserved but

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the 2-amino group is replaced by a nonpolar substituent. Further studies aimed at elucidating the mechanism of action of these compounds and exploring their therapeutic potential are planned.

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A Carboxy-Terminus Truncated Analogue of Angiotensin II, [Sar^1]angiotensin II-(1–7)-Amide, Provides an Entry to a New Class of Angiotensin II Antagonists

Sir:

Potent antagonists to the angiotensin II (AII) receptor have traditionally been obtained by a variety of alterations in position 1 (aspartic acid), 4 (tyrosine), and 8 (phenylalanine) of the AII sequence.[1] The most potent antagonists reported belong to a class of analogues obtained by a combined substitution of Phe^8 by aliphatic amino acids and of Asp^1 by sarcosine.[2] The chemical and biological properties of saralasin, [Sar^1,Val^5,Ala^8]AII,[3] which has blood pressure lowering activity in humans, and related analogues, e.g., [Sar^1,Ile^8]AII and [Sar^1,Thr^8]AII, have been studied extensively.[4,5] A second class of antagonists differs structurally from AII by modification at the Tyr^4 residue. Sarmesin, [Sar^1,(Me)Tyr^4]AII, the prototype of this second class, is a less potent but fully competitive, reversible antagonist.[6]

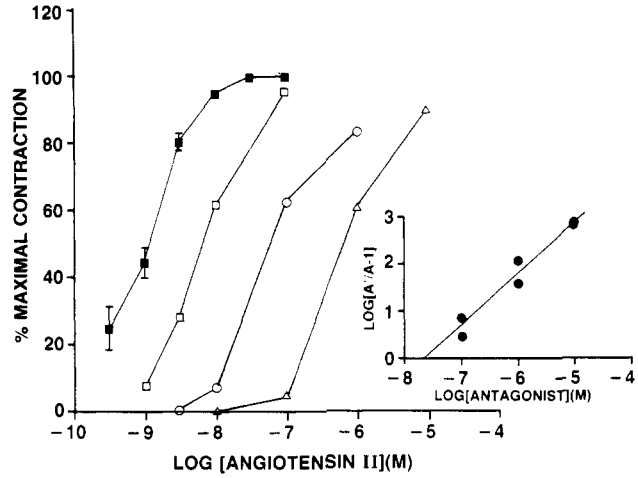

Figure 1. Concentration–response curves for angiotensin II in isolated rabbit aortic rings from a representative experiment in the absence (■) and presence (□) of 10^{-7} M, (○) 10^{-6} M, and (△) 10^{-5} M [Sar^1]AII-(1–7)NH_2. pD_2 for angiotensin II = 8.98 ± 0.07 (n = 6). Inset: Schild plot of all data with m = 1.08 ± 0.12 and pA_2 = 7.63 ± 0.20.

Despite the development of a significant number of related peptidic AII antagonists over the last 20 years, comparatively little information has appeared in the literature regarding their structure–activity relationships and in particular their chain-length requirements. This is perhaps due to the structure of saralasin-like compounds for which the antagonistic activity is directly linked to the presence of specific amino acids at both termini.

One strategy to design peptidic hormone antagonists is based on the concept that a peptide hormone is composed of distinct binding and activating components.[7] Synthesizing the binding component alone should produce a fragment which binds to the receptor without activating second-messenger systems and initiating biological response. In the present study, we used this strategy to design [Sar^1]angiotensin II-(1–7)-amide ([Sar^1]AII-(1–7)-NH_2, 1) as the most rational probe based on the hypothesis that residues 1–7 define the specificity, intensity, and duration of action of the biological effect, while the nature of residue 8 invokes agonist or antagonist activity.[8,9] In addition Sar was introduced in position 1, a substitution known to increase the potency in other AII analogue series.

[Sar^1]AII-(1–7)NH_2 (1) and sarmesin were prepared by solid-phase synthesis[10] with the aid of an Applied Biosystems Inc. Model 430A peptide synthesizer. Purification was by preparative, reverse-phase, high-performance liquid chromatography on a C_{18} bonded silica gel column. The

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