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NEW ANTITUMOR AGENTS FROM INHIBITORS OF FOLATE-DEPENDENT ENZYMES

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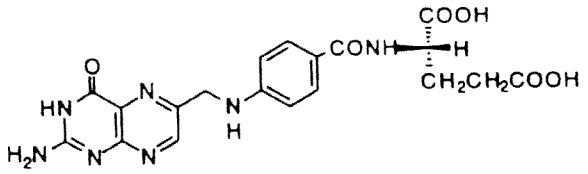
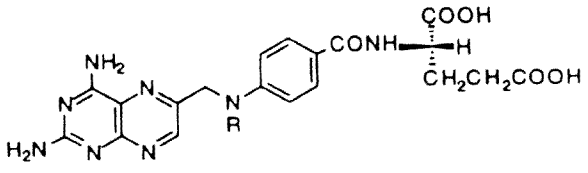
This lecture summarizes our recent investigations in folate analogue chemistry, which have resulted in the synthesis of several extremely promising chemotherapeutic agents for the treatment of solid tumors. Much of this work has been published previously; this review lecture is concerned with some of the chemical developments of interest in the field of heterocyclic chemistry.

Present-day cancer chemotherapy had its beginnings in the late 1940s, with the discovery that aminopterin and methotrexate (MTX), synthesized as potential antimetabolites of the recently discovered cofactor folic acid, induced remission of acute lymphoblastic leukemia [1-4]. It was later found that the biological basis for this cytotoxicity was inhibition of an enzyme called dihydrofolate reductase (DHFR). This enzyme regenerates tetrahydrofolic acid from dihydrofolic acid and thus maintains a constant cellular supply of this critical cofactor. There is a family of tetrahydrofolate coenzymes that play critical roles in a number of cellular one-carbon transfer reactions essential for cell growth and cell division. These include the *de novo* biosynthesis of both purine and pyrimidine nucleotides, the conversion of homocysteine to methionine, and the interconversion of glycine and serine [5]. In the majority of these metabolic reactions, dihydrofolate reductase plays a central role in regenerating the essential tetrahydrofolate cofactor, with the consequence that inhibitors of this enzyme have the potential to be potent antiproliferative agents, since they interfere with cellular processes essential for cell growth. The extreme toxicity of MTX and other DHFR inhibitors is due in part to the consequences of inhibiting these critical processes in normal cells as well as in tumor cells. The clinical usefulness of DHFR inhibitors such as MTX has been severely limited by this general cytotoxicity, despite the fact that these compounds have been in clinical use for more than 40 years [5-10].



Folic Acid



R = H Aminopterin
R = CH_3 Methotrexate (MTX)

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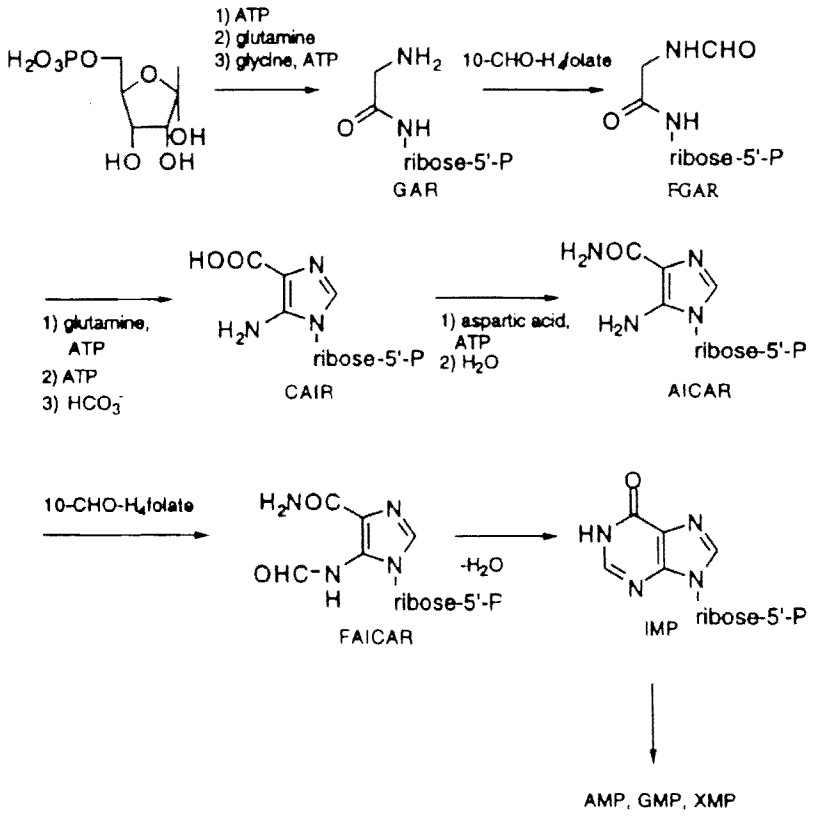
There is another centrally important metabolic process, however, which utilizes a folate-derived cofactor but which does not involve DHFR. This is *de novo* purine biosynthesis, which is outlined in Scheme 1. It will be noted that there are two one-carbon transfer reactions involved in this pathway, both of which utilize a folate cofactor (10-formyl-5,6,7,8-tetrahydrofolic acid). The first is the conversion of glycinamide ribonucleotide to its N-formyl derivative, thus introducing the eventual C-8 atom of the purine ring. The second one-carbon transfer involves formylation of an aminoimidazole intermediate; it is this second formyl grouping which becomes C-2 in the eventual purine ring. The first of these one-carbon transfer reactions is expanded on Scheme 2. The folate cofactor transfers its N-10 formyl group to glycinamide ribonucleotide in a reaction mediated by an enzyme called glycinamide ribonucleotide formyltransferase (GAR FTase), *without concomitant oxidation of the cofactor to its dihydro derivative*. Thus, regeneration of the cofactor only involves reformylation and does not require reduction by DHFR.

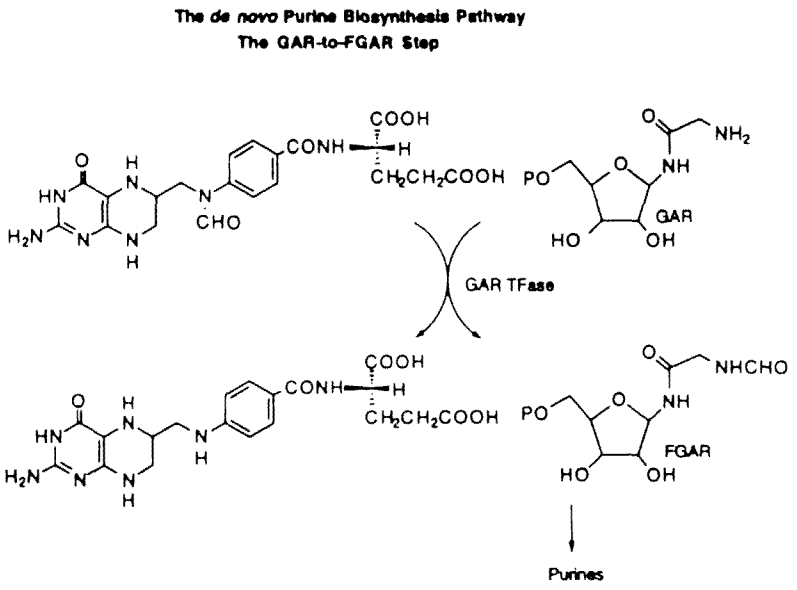
Scheme 1

The *de novo* Purine Biosynthetic Pathway

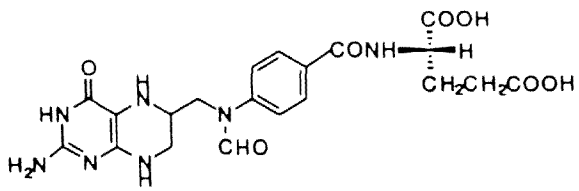


About 12 years ago, our research group at Princeton started a program aimed at the preparation of possible analogues of aminopterin and MTX which might be more selective in targeting tumor cells over normal cells. This was a rather empirically driven project, where we hoped that increasing the lipophilicity of our target inhibitors by removing, for example, some of the ring nitrogen atoms of the pterin ring system might result in greater cell permeability, or might take advantage of conceivable differences between the DHFR in normal cells and that in tumor cells. In the event, we prepared a number of deaza derivatives of aminopterin, including one in which we had replaced with carbon atoms both the nitrogen atom at position 5, and the NH unit in the side-chain at position 10. The resulting compound, 5,10-dideazaaminopterin, proved to be a potent inhibitor of DHFR and, as expected, was also a potent cytotoxic agent. Unfortunately, it also proved to be just as toxic to normal cells as aminopterin or MTX, and thus was not a promising chemotherapeutic agent [11].

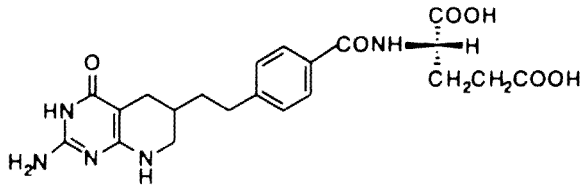
Scheme 2



At the same time, however, we had prepared a derivative of this compound in which the 4-amino group had been hydrolyzed to a lactam oxygen atom (producing a pyrimidine ring bearing the same substituents as are present in folic acid itself), and the pyridine ring had been reduced to a tetrahydro derivative. This compound proved to be an extremely effective cytotoxic agent, active against a broad range of over a dozen common solid tumors, including breast, lung, and colon cancers, as well as B-16 melanoma. Our interest in this new cytotoxic agent was intensified by an *in vitro* comparison with MTX carried out by Dr. Gerald B. Grindey of Eli Lilly and Company. It will be seen that this new compound, which we called DDATHF (5,10-dideaza-5,6,7,8-tetrahydrofolic acid), was fully effective against a range of tumors against which MTX is inactive. Furthermore, this new compound proved to be fully active against tumors which had developed resistance to MTX, presumably due to amplification of the dihydrofolate reductase gene [12, 13]. A closer examination of DDATHF revealed that it was inactive as an inhibitor of DHFR, as might have been expected from its chemical structure, and that its cytotoxic activity was a result of inhibition of the first formyl transfer reaction in *de novo* purine biosynthesis. It is worth noting at this point that the activity of DDATHF as an antitumor agent is probably a consequence of a large number of factors, which include its chemical stability to oxidation (the tetrahydropyridine ring, as contrasted with the tetrahydropyrazine ring of the natural cofactor, is relatively stable to oxidation), its inactivity against DHFR (a probable consequence of the absence of the 4-amino substituent in the pyrimidine ring), its inability to be converted to a surrogate factor (due to the absence of nitrogens at positions 5 and 10), and its otherwise striking structural resemblance to the natural folate cofactor (suggesting that its ability to enter cells probably involves pathways involving both the reduced folate and the folate binding membrane protein transport systems, as well as its ability to serve as a substrate for folylpolyglutamate synthetase (FPGS) within the cell).



10-Formyl-5,6,7,8-tetrahydrofolic Acid, the Natural Cofactor for GAR FTase



5,10-Dideaza-5,6,7,8-tetrahydrofolic Acid (DDATHF, Lometrexol)

In my lecture today, I would like to discuss some of the chemistry associated with the synthesis of DDATHF and some of its analogues, and a few of the surprises, both chemical and biological, encountered in the course of this investigation.

Our intial synthesis of DDATHF, which commenced with a condensation of α-cyanothioacetamide with 2-methyl-3-ethoxyacrolein to give 3-cyano-5-methyl-2(1H)-pyridinethione, proceeded linearly through a total of 18 steps, and was neither suitable for the preparation of the quantities of material required for clinical investigation, nor sufficiently flexible for the convenient preparation of structural analogues [14]. As a consequence, we have over the past ten years or so devised a total of eight separate synthetic approaches to this compound. Since details on many of these have already been published, I have chosen to talk about only one of them, selected to illustrate the effectiveness of palladium-catalyzed carbon–carbon coupling reactions in this area of heterocyclic chemistry. In this approach (which is one of the most direct and efficient syntheses we have devised [15]), the pyrimidine ring was constructed first, followed by annulation of a pyridine ring bearing a halogen substituent at position 6. Two successive palladium-catalyzed carbon–carbon coupling reactions were then employed, the first to add the two-carbon bridge to the pyridine ring, and the second to add the remainder of the carbon skeleton of DDATHF. The complete reaction sequence is illustrated in Scheme 3. Thus, malondialdehyde tetramethylacetal was hydrolyzed with HCl to yield the free dialdehyde, which was then brominated to give bromomalondialdehyde. This was condensed directly with 2,4-diamino-6(1H)-pyrimidinone to give 6-bromo-5-deazapterin as an extremely insoluble product which was, however, readily converted to its easily manipulated and reasonably soluble 2-pivaloyl derivative by treatment with pivalic anhydride in pyridine. Palladium-catalyzed coupling of this intermediate with trimethylsilylacetylene followed by desilylation then gave 6-ethynyl-2-pivaloyl-5-deazapterin. This compound was then coupled, utilizing a second palladium-catalyzed reaction, with diethyl 4-iodobenzoyl-L-glutamate. Simultaneous catalytic reduction of the triple bond and the pyridine ring, followed by removal of protecting groups, gave DDATHF.

It will be noted that the above (non-enantioselective) reduction of the pyridine ring gave DDATHF as a mixture of two diastereomers. These two diastereomers were separated by a tedious resolution with camphor-D-sulfonic acid; the 6(R) diastereomer, which possesses the same absolute configuration at C-6 as found in the natural tetrahydrofolate cofactor, is now known as Lometrexol and is well advanced in Phase II clinical trials for the treatment of solid tumors.

A totally different synthetic strategy was used to prepare homoDDATHF, an analog possessing a stretched 3-carbon bridge between the benzoylglutamate and 5-deazapterin moieties, which was of interest because of its increased flexibility as compared with DDATHF itself. In this synthesis (which was also utilized for an independent preparation of DDATHF), a palladium-catalyzed carbon–carbon coupling between 1-butyne-4-ol and methyl 4-bromobenzoate, followed by catalytic reduction of the resulting acetylene, gave the 4-aryl-1-butanol shown in Scheme 4. This was oxidized with pyridinium chlorochromate to the corresponding aldehyde, which was then condensed with malononitrile. The resulting dicyanomethylene derivative was formylated with triethyl orthoformate. The product of this formylation was condensed with 2,4-diamino-6(1H)-pyrimidinone to yield directly and in good yield the 6-substituted 5-deazapterin. A series of standard transformations yielded homoDDATHF, again as a mixture of two diastereomers [16].

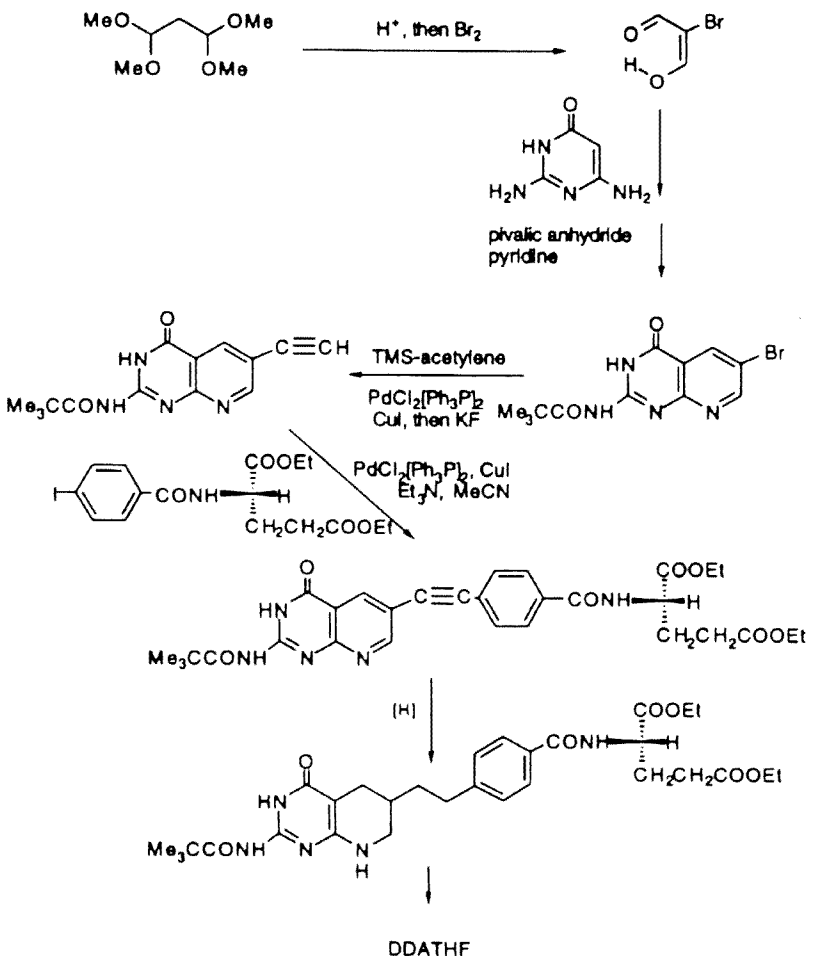
The reason for my selection of this alternative synthetic strategy for illustration lies in the above particularly effective ring annulation step, which illustrates a concept which we have termed "carbonyl group activation."

Its essential feature is the utilization of the formyl derivative of the malononitrile Knoevenagel product A (Schemes 4 and 5) as a highly activated malondialdehyde equivalent. The first step in the ring annulation sequence (Scheme 5) is a Michael addition of the nucleophilic 5-position of 2,4-diamino-6(1H)-pyrimidinone to the activated malondialdehyde equivalent A. This is followed by elimination of ethanol, a second (but intramolecular) Michael addition, and final aroma-

Scheme 3

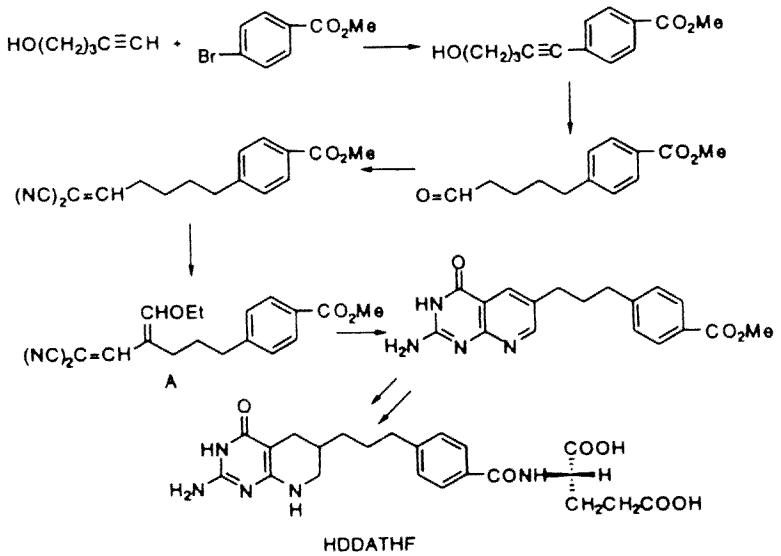
A New Synthesis of DDATHF

The Palladium-mediated C-C Coupling Approach



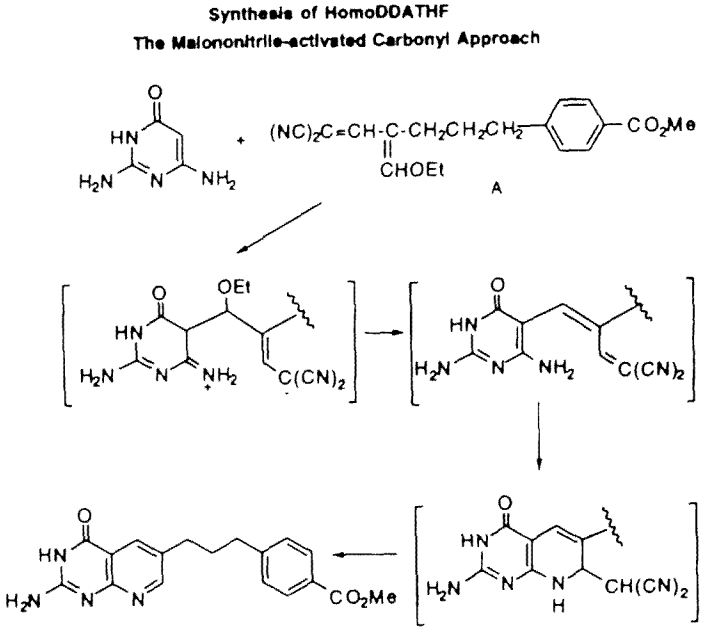
Scheme 4

Synthesis of HomoDDATHF (HDDATHF)



tization through loss of malononitrile. Each of the above steps (the initial Michael reaction at C-5 of the pyrimidinone, loss of ethanol, the intramolecular Michael reaction which results in ring closure, and the terminal aromatization step) is facilitated by the replacement of an aldehyde carbonyl CH=O by the much more electron-withdrawing $CH{=}C(CN)_2$ grouping. Furthermore, in principle (but not actually in practice thus far), malononitrile can be considered to be a catalyst for the entire sequence of reactions.

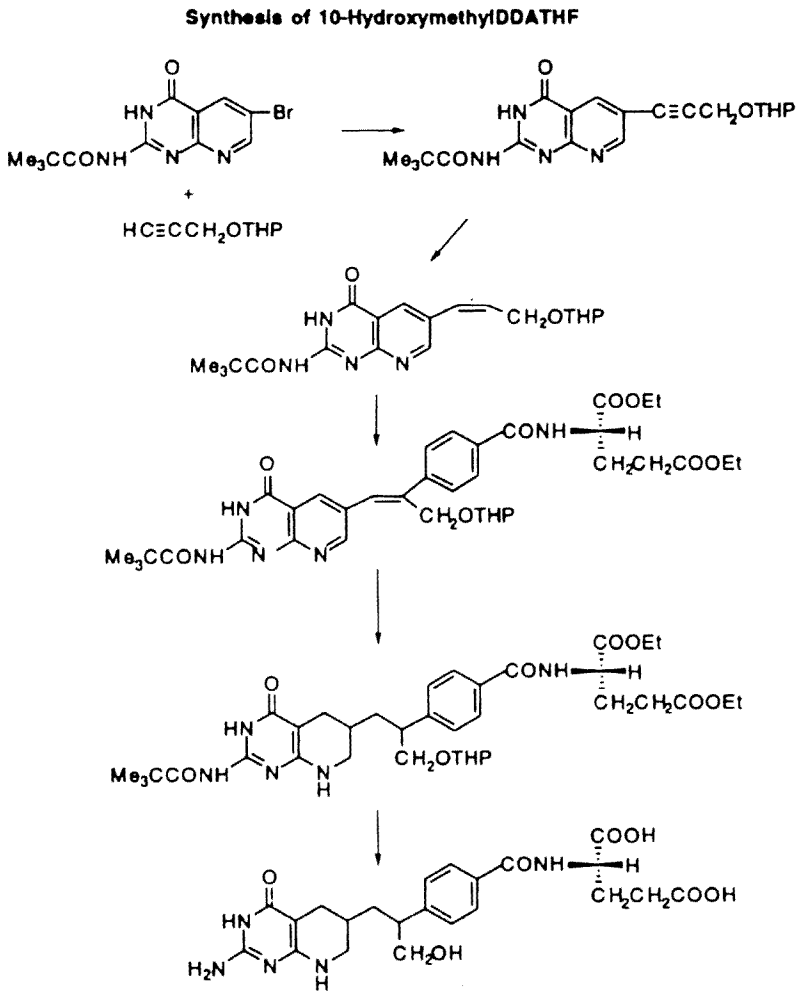
Scheme 5



One obvious feature of the structure of DDATHF is the absence of a substituent at the bridge 10-position which might resemble the N-formyl group of the natural cofactor for the GAR FTase-mediated formylation reaction. One of our earlier objectives in this synthetic program, therefore, became the synthesis of an analogue bearing a substituent at C-10 which would either mimic the N-formyl group of the natural cofactor, or preferably serve as a "transition-state analog" for the formyl transfer step itself. We chose a 10-hydroxymethyl substituted DDATHF, and its synthesis is shown in Scheme 6. As you will note, once again palladium-catalyzed carbon–carbon coupling plays a key role in the construction of this target molecule. Thus, propargyl alcohol, protected as its THP derivative, was condensed under palladium catalysis with the same 6-bromo-2-pivaloyl-5-deazapterin utilized as an intermediate in the DDATHF synthesis (see Scheme 3). Reduction of the resulting alkyne under Lindlar conditions to the *cis*-alkene was carried out using hydrogen and palladium/barium sulfate, with freshly distilled synthetic quinoline as the catalyst poison. The resulting *cis*-olefin was then regiospecifically coupled with diethyl 4-iodobenzoyl-L-glutamate in the presence of palladium acetate, and the coupled product was reduced using palladium oxide and glacial acetic acid to avoid hydrogenolysis of the allyl alcohol. 10-HydroxymethylDDATHF was then obtained by removal of all of the protecting groups [17].

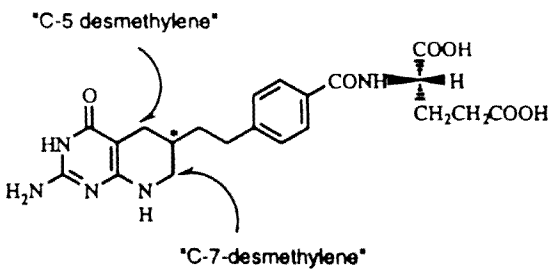
It will be noted that four diastereomers are produced in the above process. Even so, preliminary biological evaluation of this mixture revealed extremely potent cytotoxic activity. We had earlier shown that 10-methylDDATHF, which was similarly produced as a mixture of four diastereomers, was some ten-fold less active than this mixture of diastereomers of 10-hydroxymethylDDATHF. Significantly, however, we had been able to separate these four diastereomers of 10-methylDDATHF, and to show that all of its cytotoxic activity was due to only one of the four diastereomers [18]. If this should also prove to be the case for 10-hydroxymethylDDATHF, we would have in hand the most active analog of DDATHF which we have prepared thus far. Because of the formidable separation difficulties, however, we have not yet pursued this interesting analogue further at the present time.

Scheme 6



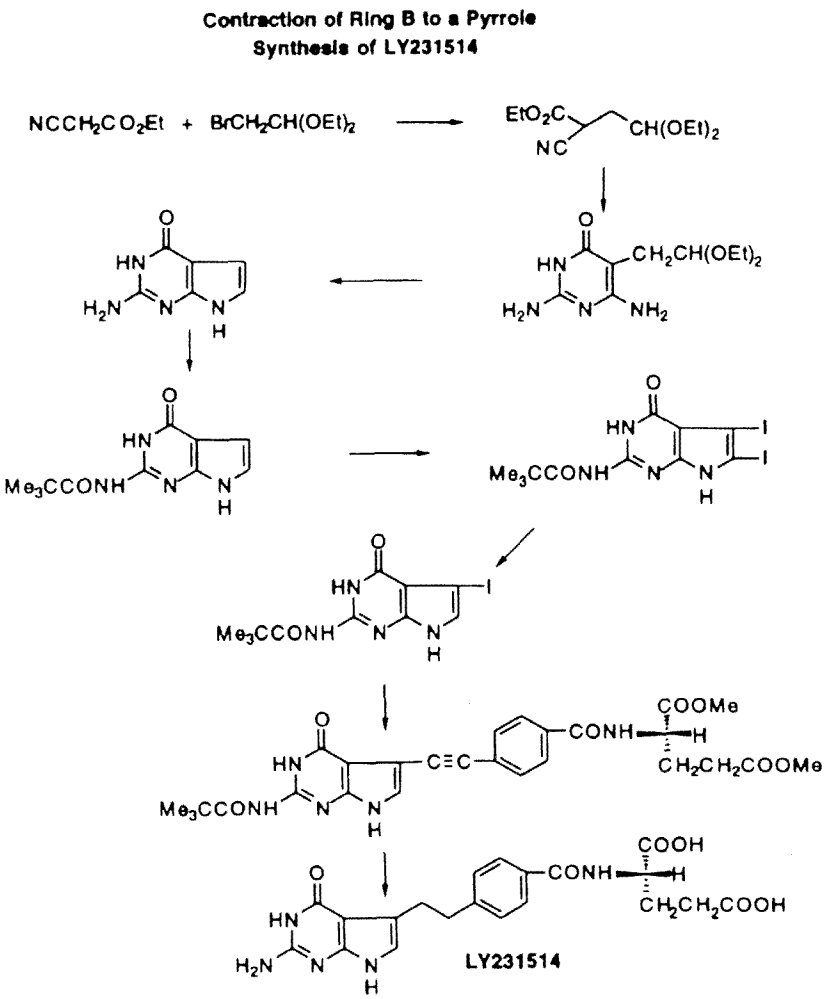
A problem encountered with all of the compounds thus far described is generation of the C-6 prochiral center in racemic form as a consequence of the non-enantioselective pyridine reduction step. Since all attempts to carry out this reduction in an enantioselective manner, using chiral catalysts, or chiral auxiliaries, were unsuccessful, we explored alternative strategies aimed at eliminating this C-6 chiral center in its entirety. For example, two different series of "open-chain" analogues were prepared by deletion of the C-5 methylene group [19], or by deletion of the C-7 methylene group [20-23] (see Scheme 7).

Scheme 7



Very promising *in vitro* (but not *in vivo*) activity was observed with the C-7 desmethylene analogues; derivatives belonging to the C-5 desmethylene series were almost completely devoid of cytotoxic activity. However, a third strategy was available in principle to eliminate the potential C-6 chiral center; i.e., one could convert the sp^3 carbon at C-6 of DDATHF to an sp^2 carbon. Since previous studies in our laboratories had shown that an N—H grouping at C-4 of the pyrimidine ring was mandatory for reactivity against GAR FTase, and a rigid bicyclic system with the same pyrimidine ring substituents as present in DDATHF (and in folic acid) was also required, we were left with a 5-substituted pyrrolo[2,3-*d*]pyrimidine. This intriguing target compound was prepared as shown in Scheme 8. Alkylation of ethyl cyanoacetate with bromoacetaldehyde diethylacetal was followed by guanidine ring closure to give 2,4-diamino-6(1H)-pyrimidinone bearing an acetaldehyde diethylacetal substituent at position 5.

Scheme 8

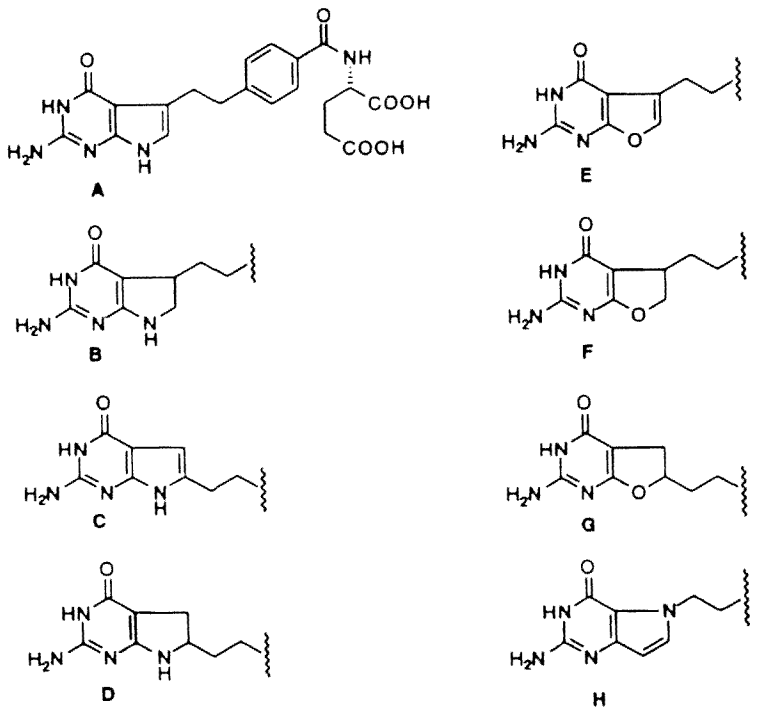


Acid hydrolysis then led directly to 7-deazaguanine. This key precursor was pivaloylated, and the resulting soluble intermediate was subjected to iodination with 2,2 equivalents of N-iodosuccinimide. The resulting 7,8-diiodo derivative could be selectively mono-deiodinated with zinc and acetic acid to the desired 2-pivaloyl-7-iodo-7-deazaguanine. This compound was then coupled with dimethyl 4-ethynylbenzoyl-L-glutamate (itself prepared by palladium-catalyzed coupling of dimethyl 4-iodobenzoyl-L-glutamate with trimethylsilylacetylene, followed by desilylation), and the resulting acetylene was successfully reduced, without reduction of the pyrrole ring, using hydrogen and 3% palladium-on-charcoal catalyst in a mixture of methylene chloride and methanol.

The target pyrrolopyrimidine was then obtained by removal of the protecting groups [24].

This product, which we now call LY231514 as a consequence of our collaboration with Eli Lilly and Co. in its development, proved to be an extremely active inhibitor of tumor growth, both *in vitro* and *in vivo*. To our great surprise, however, its activity as a cytotoxic agent was largely due to inhibition of thymidylate synthase (TS), not GAR FTase. Thus, this compound is responsible for the inhibition of the biosynthesis of thymidylate (and thus DNA) rather than purine biosynthesis. It will also be noted that, since this material is a single enantiomer, there is no need for a resolution step, and its synthesis in quantity is thus substantially simpler than that of Lometrexol. LY231514 is now in Phase II clinical trials for the treatment of solid tumors.

Ring B Analogues of LY231514



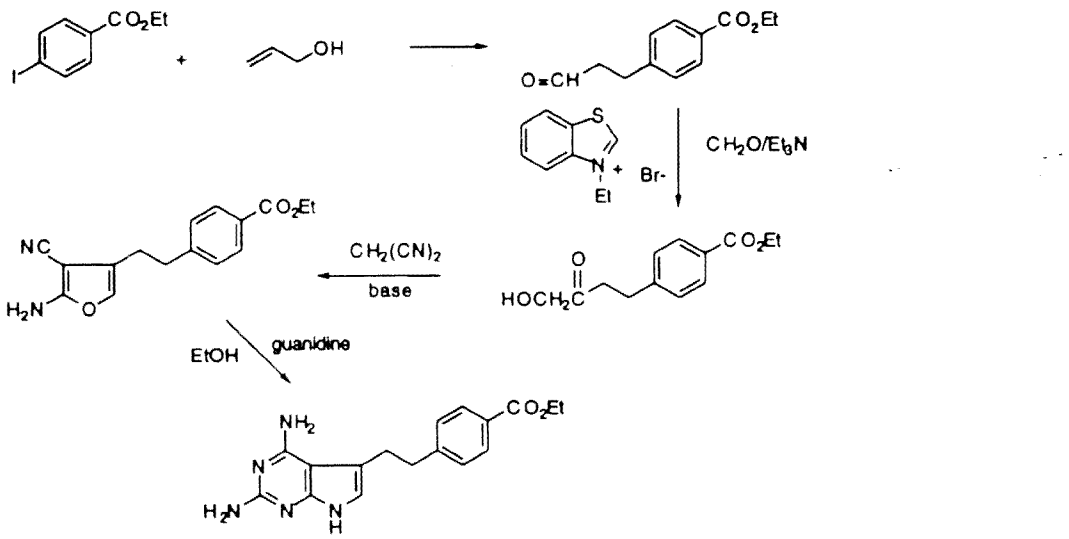
We have prepared a large number of analogues of LY231514 in an attempt to delineate the structural requirements for TS inhibitory activity in this unique series. A few of the compounds prepared and evaluated are illustrated below. Compound B is the dihydro derivative of LY231514 (compound A), and it could be considered as a DDATHF analogue in which the C-5 methylene group had been excised, and the 5-position of the pyrimidine ring connected directly to the carbon bearing the ethano bridge linkage. However, compound B also proved to be a TS inhibitor; it was not a GAR FTase inhibitor. Compound C represents an analogue in which two carbons now separate the ethano bridge from the C-5 position of the pyrimidine ring (as is the case in DDATHF), but the bridge is joined to an sp^2 rather than to an sp^3 carbon. Biological evaluation revealed that this compound was completely inactive as a cytotoxic agent. By contrast, however, compound D (the dihydro derivative of C) now represents a DDATHF analogue in which the C-7 methylene group of DDATHF has been deleted, and C-6 of DDATHF has been connected directly to the pyrimidine-4-NH grouping. This analogue (as a mixture of two diastereomers, of course) once again exhibited excellent antitumor activity. *Remarkably, however, it proved to be an inhibitor of GAR FTase, not TS!* It is noteworthy that very minor structural changes can completely switch the enzymatic target of inhibition of these derivatives from GAR FTase to TS. In order to explore the consequence of replacing the pyrrole N—H grouping by other heteroatoms, we have prepared a number of additional 6-5 systems, some of which are also illustrated above. The furan analogue, compound E, proved to be inactive, as was its dihydro derivative F. The positional isomer G was also inactive. Compound H is a derivative of 9-deazaguanine in which the side-chain is now attached to nitrogen and the 9-NH grouping of LY231514 is replaced by an sp^2 carbon atom [25]. This compound also proved (rather surprisingly) to possess some activity, although it was several orders of magnitude less active than LY231514.

These results serve to emphasize that a hydrogen-bonding donor at (purine) position 9 seems to be required for maximum cell growth inhibitory activity.

In any investigation of this type, unusual chemical reactions are almost invariably observed, and I would like to close with an example of an unexpected ring transformation reaction which may prove to be of considerable synthetic utility. In our first approach to the furan analogue E, we attempted to utilize a strategy which is apparent from Scheme 9. Palladium-catalyzed carbon−carbon coupling of allyl alcohol with ethyl 4-iodobenzoate led directly to the β-arylpropionaldehyde shown. This compound was smoothly converted in a single step to the corresponding α-hydroxyketone by a benzoin condensation employing N-ethylbenzothiazolium bromide, formaldehyde, and triethylamine.

Scheme 9

Attempted Synthesis of the Furan Analog of LY231514
A Facile Ring Transformation/Annulation Sequence

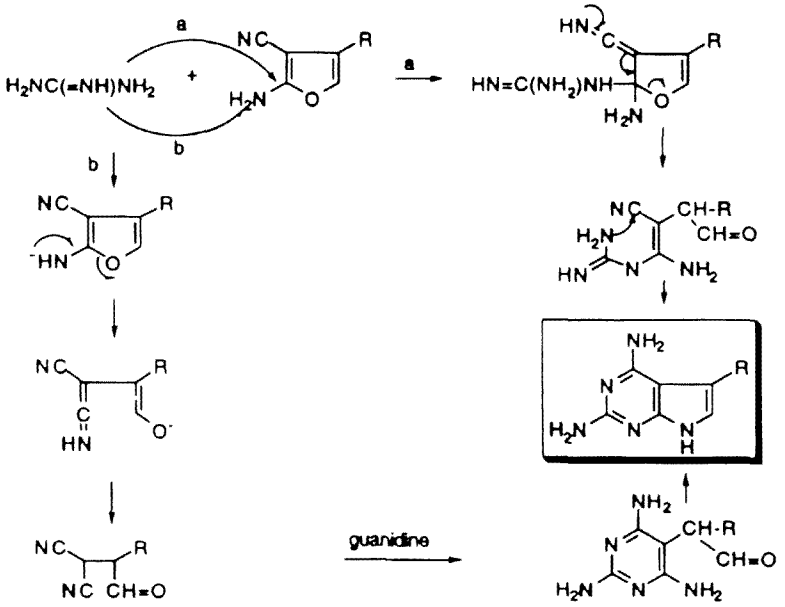


Condensation with malononitrile yielded a 2-amino-3-cyano-4-substituted furan which we anticipated could be subjected to the classic guanidine/*o*-aminonitrile pyrimidine ring annulation reaction to give a 2,4-diaminofuro[2,3-*d*]pyrimidine, which we then planned to convert on to our target furo[2,3-*d*]pyrimidine analogue of LY231514. To our surprise, however, the product of the reaction of the above furan *o*-aminonitrile with guanidine proved to be a 2,4-diaminopyrrolo[2,3-*d*]pyrimidine. This unexpected furan-to-pyrrolopyrimidine transformation was fairly general with amidines other than guanidine. We have, for example, successfully utilized the guanidine-initiated transformation [26] in a very efficient synthesis of TNP-351, a 2,4-diaminopyrrolo[2,3-*d*]pyrimidine DHFR inhibitor developed by Takeda Laboratories in Japan [27].

Several plausible mechanisms for this amidine-induced ring transformation/ring annulation reaction can be suggested (Scheme 10).

For example, initial Michael addition of the amidine to the 2-position of the 2-amino-4-cyanofuran could be followed by a reverse Michael reaction involving cleavage of the C—O bond of the furan ring. This would generate an open-chain carbonyl derivative which then recyclizes to yield an intermediate pyrrole in which the pyrrole N—H grouping arises from the original 2-amino group of the starting furan *o*-aminonitrile. Ring closure of the pyrimidine ring is a consequence of a normal amidine cyclization upon the *o*-situated nitrile. Alternatively, one could suppose deprotonation of the acidic 2-amino group of the starting 2-amino-3-cyanofuran, followed once again by C—O cleavage to generate an intermediate ketenimine which, by prototropic rearrangement, would give a substituted malononitrile. Reaction with the amidine would then result in ring closure to a 4,6-diaminopyrimidine bearing an acetaldehyde substituent at position 5. Final ring closure would then yield the fused pyrrolopyrimidine. At this point we have no evidence which would distinguish between these alternative mechanisms.

Scheme 10



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