THE CHEMISTRY OF DDATHF (5,10-DIDEAZA-5,6,7,8-TETRAHYDROFOLIC ACID) AS ANTITUMOR AGENT *

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Abstract - Over the past 40 years, big efforts have been devoted to the development of novel folate antimetabolites. All of the potent antifolates have reportedly been inhibitors of dihydrofolate reductase (DHFR). In 1985, Taylor and et al. reported the synthesis of 5,10-dideaza-5,6,7,8-tetrahydrofolic acid, DDATHF, which exhibits broad and selective antitumor activity as an inhibitor of glycinamide ribonucleotide formyltransferase (GARFT). DDATHF is a close analog of tetrahydrofolic acid, differs only by replacement of the 5- and 10- position nitrogen atoms by carbon. It may exist in two diastereomeric forms, differing in configuration at carbon 6. Both diastereomers of DDATHF are potent inhibitors of cell growth in culture. DDATHF is currently in Phase II clinical trials.

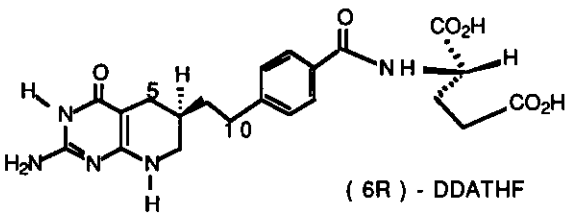
I. INTRODUCTION

The history of antimetabolite cancer chemotherapy has begun when aminopterin, and methotrexate (MTX), both inhibitors of folate metabolism, were found to induce remission of acute lymphoblastic leukemia .[1] The clinical record with MTX,which has been compiled as an antineoplastic and immunosuppressive drug testifies to the value of folate antimetabolites as antiproliferative agents.[2] All of the potent antifolates reported thus far have been inhibitors of dihydrofolate reductase (DHFR) or thymidylate synthase (TS), and none has supplanted MTX in clinical usefulness. However,the extreme toxicity of MTX[3] and its lack of effectiveness against most human tumors [4] have limited the utility of this drug. Furthermore, development of resistance to methotrexate by tumor cells remains a stubborn problem .[5]

Recently, Taylor *et al.* reported the synthesis and preliminary evaluation of a new class of tetrahydrofolate analogs, which were designed as inhibitors of folate metabolism other than DHFR .[6-9] The lead member of

*Dedicated with affection and respect to Prof.Dr. Edward C. Taylor on the occasion of his 70 th birthday.

this series is 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF),whose structure is shown in Figure I. The nitrogen atoms at 5- and 10-positions of DDATHF are essential participants in all of the 1-carbon transfers and cofactor interconversions of reduced folate metabolism, and DDATHF is structurally precluded from serving as a substrate in any of these reactions. It possesses extraordinary and selective antitumor activity. Its therapeutic index and its broad spectrum of activity against a variety of murine solid tumors and human colon xenografts in mice are unrivaled among known antitumor agent. [6-13]



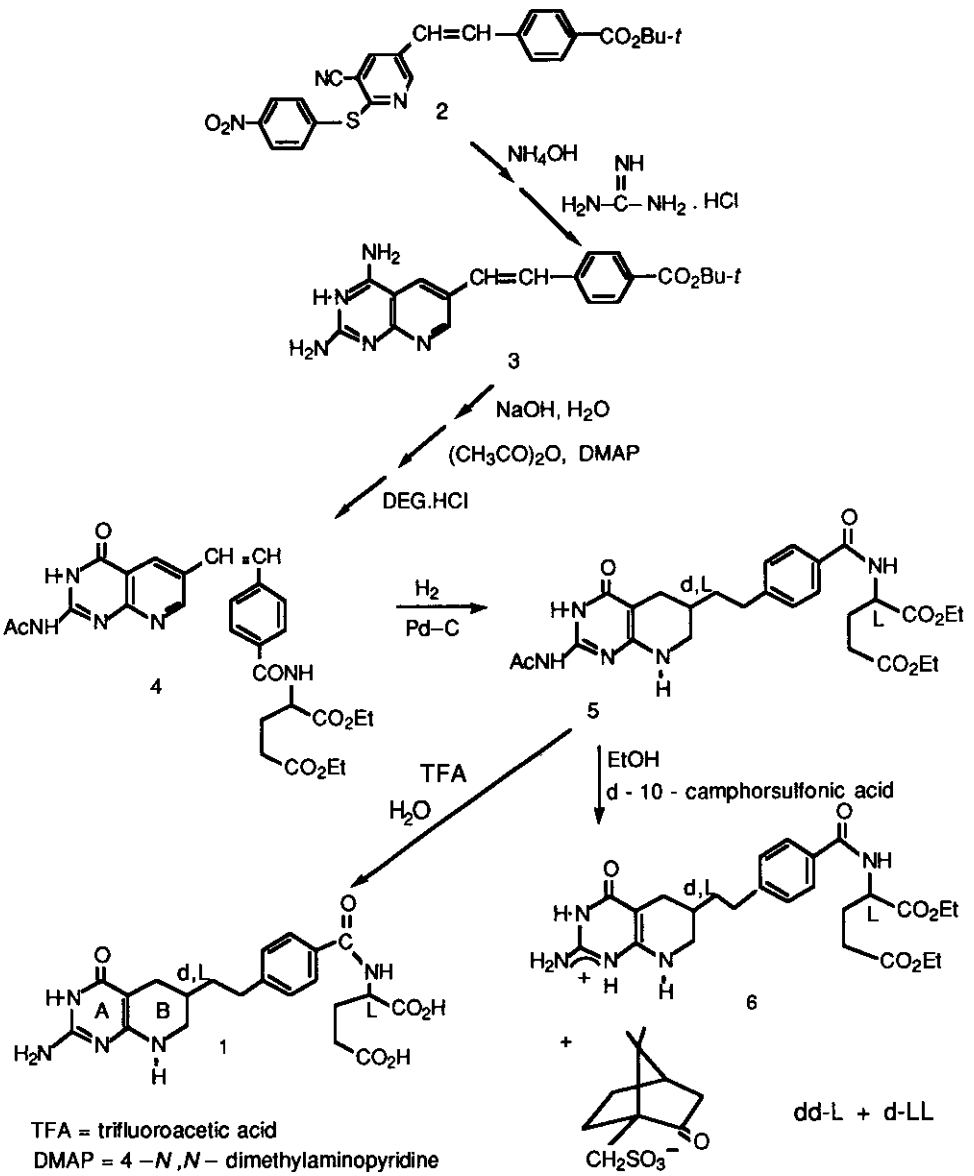
(6R) - DDATHF

Figure I.

The current level of interest in the synthesizing and biological evaluation of DDATHF and analogs is indicated by the increasing number of publications in this area from day to day. DDATHF has recently been renamed "Lometrexol", is in world-wide Phase II clinical trials. In this review, primary emphasis will be on the synthesis of DDATHF and analogs, also recent reports on intracellular metabolism of DDATHF isomers will be given.

2.SOME DIFFERENT SYNTHETIC PATHWAYS TO 5,10-DIDEAZA-5,6,7,8-TETRAHYDROFOLIC ACID

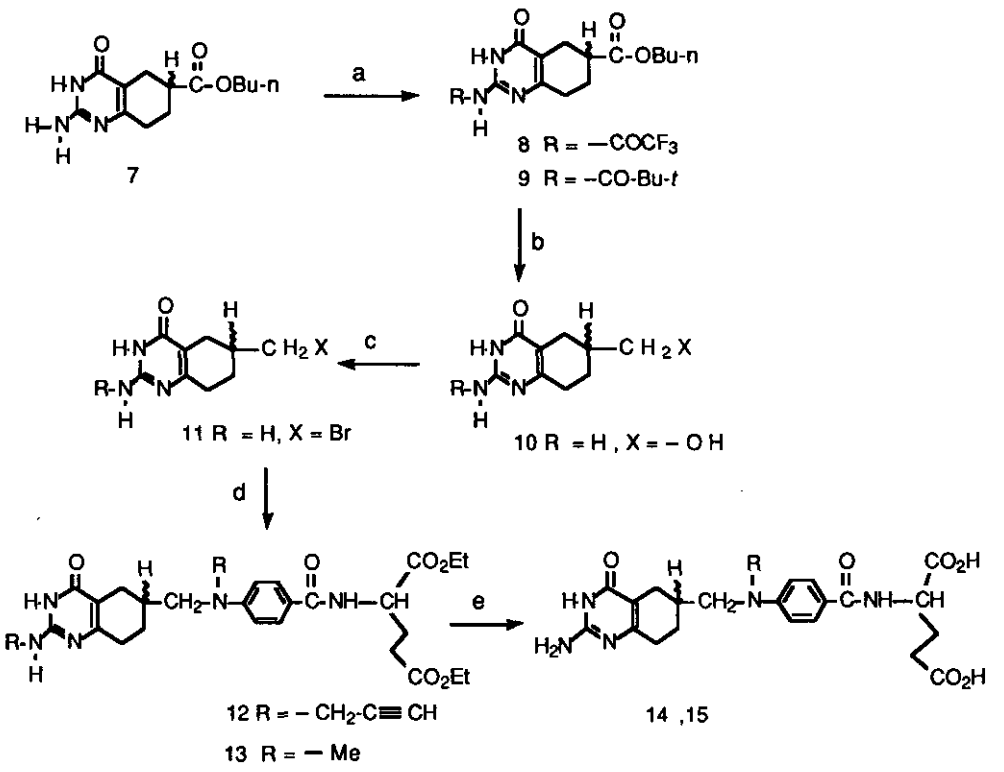
The first synthesis of DDATHF was accomplished by Taylor *et al.* [14] in 14 steps from thiocyanoacetamide and β-ethoxymethacrolein. 3-Cyano-5-methyl-2(1*H*) - pyridinethione was initially formed.[10] A Wittig condensation of its derivative with *t*-butyl-*p*-formylbenzoate gave **2**. Guanidine cyclization of **2** produced pyrido[2,3-*d*]pyrimidine **3**, followed by hydrolysis and acetylation. Coupling with diethyl L-glutamate (DEG.HCl) afforded **4**. Catalytic reduction of **4** followed by hydrolysis of both the acetyl and ester functionalities to give DDATHF **1** (Scheme I). Reaction of **5** with *d*-10-camphorsulfonic acid gave a mixture (dd-Land d-LL) of salts **6**, separated by fractional crystallization. These diastereomers were referred as "A" and "B", their absolute configurations have not been determined.[9] Biochemical studies on each diastereomer suggested that both of them inhibit the same target enzyme and the configuration at carbon 6 has only a minor effect on the growth inhibitory activity against tumor cells in culture.



Scheme I.

Related to synthesis of DDATHF analogs, Nair and his co-workers have synthesized N^{10}-propargyl-5,8-dideaza-5,6,7,8-tetrahydrofolic acid as shown in Scheme II .[15-17] On the synthesis, 2-amino-6-carbo-n-butoxy-4-hydroxy-5,6,7,8-tetrahydroquinazoline **7** was converted to 2-trifluoroacetyl and 2-trimethylacetyl derivatives **8** and **9** respectively .[18] Both of these compounds were easily reduced with superhydride, during

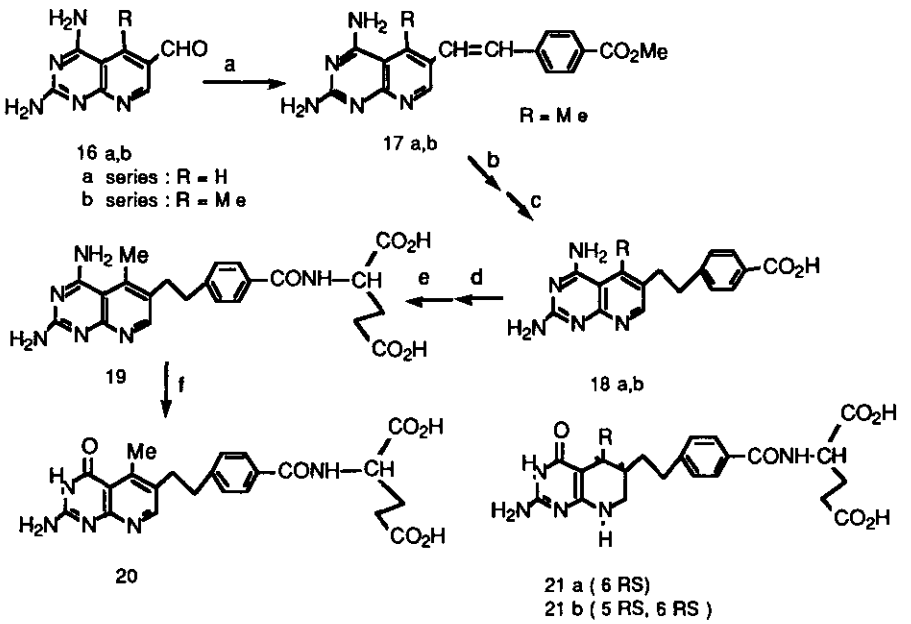
the reduction, trifluoroacetyl group of compound **8** was removed, and the alcohol **10** was isolated. Reaction of compound **10** with HBr gave 6-bromomethyl derivative **11**. Compound **11** was reacted with diethyl [*p*-(*N*-propargylamino)benzoyl]-L-glutamate or diethyl [*p*-(*N*-methylamino)benzoyl]-L-glutamate and then was hydrolyzed to target compounds **14,15** respectively. These results showed that presence of the propargyl group at N^{10} in folate analogs is necessary to create favorable binding interactions with TS.



a)($CF_3CO)_2O$ or Et_3N , *t*- Bu-C(=O)-Cl , dioxan b) Li (Et)$_3$ BH , THF c) 49 % HBr, toluene d) diethyl [4 -(*N* - propargylamino)benzoyl] -L-glutamate or diethyl [*p* - (*N* - methylaminobenzoyl] - L-glutamate MgO , DMAc e) 0.1 N NaOH , MeCN

Scheme II.

Piper *et al.*[19] have developed an alternative synthesis of 5,10-dideazatetrahydrofolic acid and its methyl analogue (Scheme III). They reviewed that modifications of classical antifolate structure at positions 5 and 10 might lead to new antifolates with enhanced selectivity of antitumor action .[20-22] Both aldehydes **16 a,b** were converted to 9,10-ethenyl precursors by Wittig condensation .[23] Hydrogenation of **17b** was followed by hydrolysis to give compound **18b**. Coupling of **18b** with dimethyl L-glutamate (DMG) by using isobutyl chloroformate and hydrolysis of diester gave compound **19**. Hydrolytic deamination of compound **19** afforded 5-methyl-5,10-dideazafolic acid **20**.They have also synthesized 5-methyl-5,10-dideazatetrahydrofolic acid **21a,b** by using same synthetic pathway. Compounds **20** and **21a,b** were transported into cells more efficiently than methotrexate.

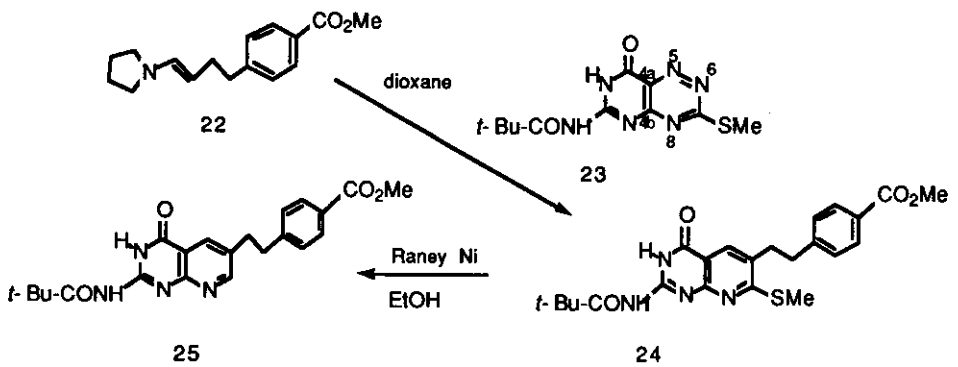


a) [methoxycarbonylbenzyl]triphenylphosphoniumbromide, NaOMe,DMF b) H_2 / Pd - C, DMF c) 1 N NaOH, Me_2SO d) Et_3N , dimethyl L - glutamate. HCl or diethyl L- glutamate. HCl, DMF, i -BuOCOCl , e) 1 N NaOH, Me_2SO f) 1 N NaOH, 1 N HCl

Scheme III.

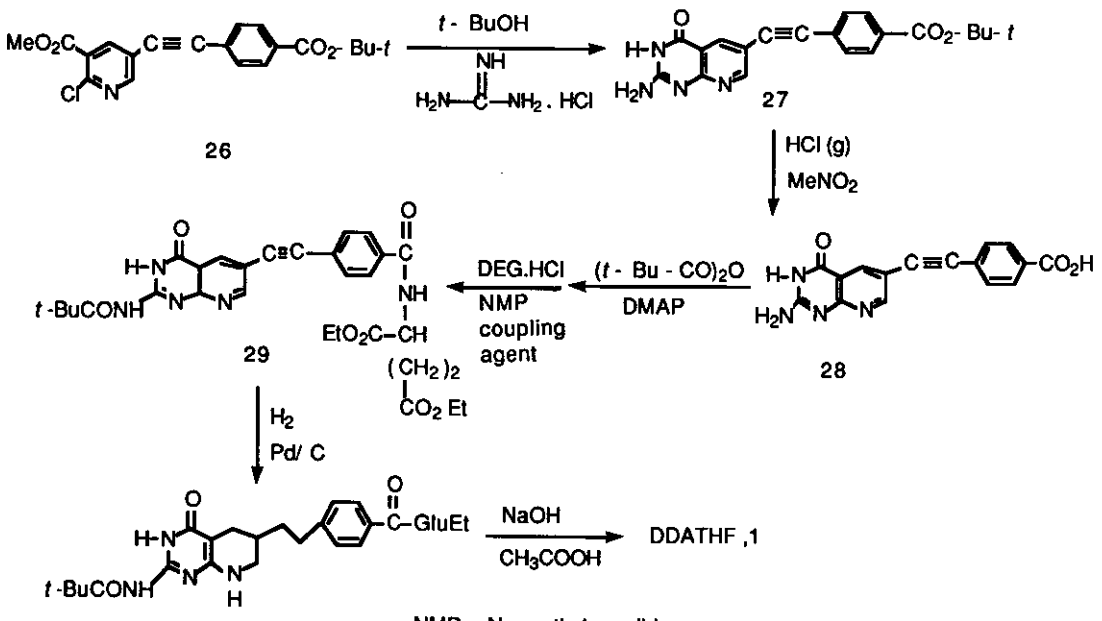
Taylor and co-workers [24] have prepared dideazapteroate **25** by an inverse electron demand Diels-Alder reaction [25] between pyrrolidine enamine **22** and 6-azapterin **23**. Heating of **22** with 2-*N*-pivaloyl -7-methylthio- 6-azapterin **23** in anhydrous dioxan gave the Diels- Alder product **24** in a modest yield.The

regiochemistry of this cycloaddition product **24** was confirmed by Raney Nickel desulfurization and methyl 2*N*-pivaloyl-5,10-dideazapteroate **25** was obtained (Scheme IV). Compound **25** has been used as a key intermediate for the preparation of DDATHF .[8-10,26,27]



Scheme IV.

Taylor and Wong[28] have reported efficient Palladium-effected synthesis of DDATHF. A key feature of the synthesis was a double exploitation of a Pd catalyzed carbon-carbon [9,10,29] bond coupling reaction.

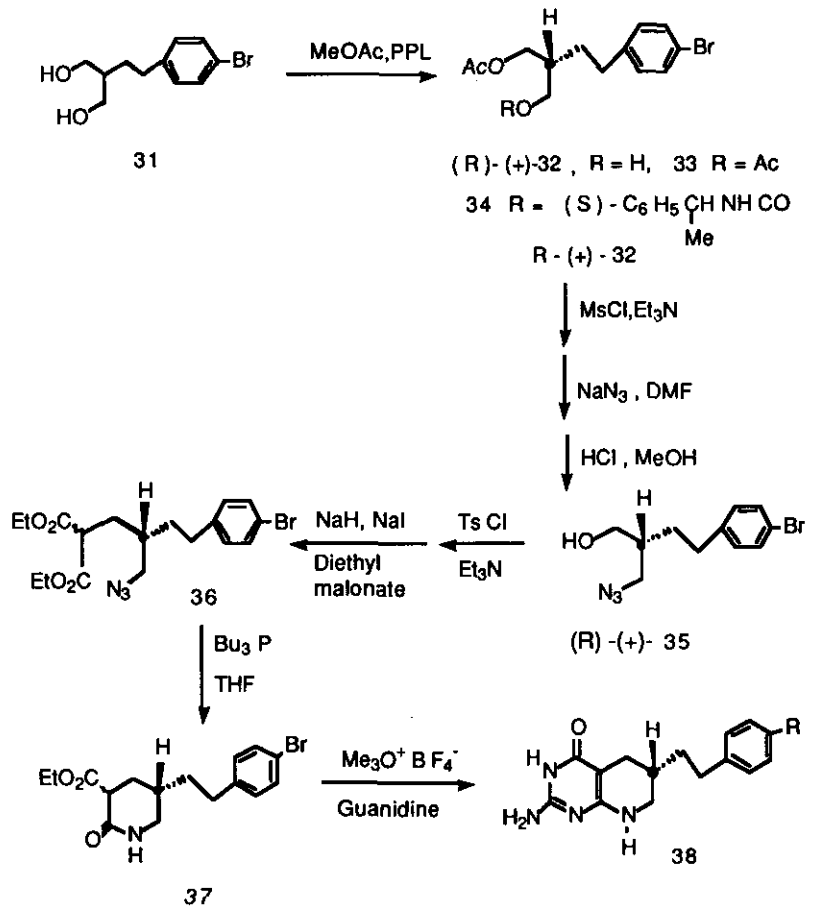


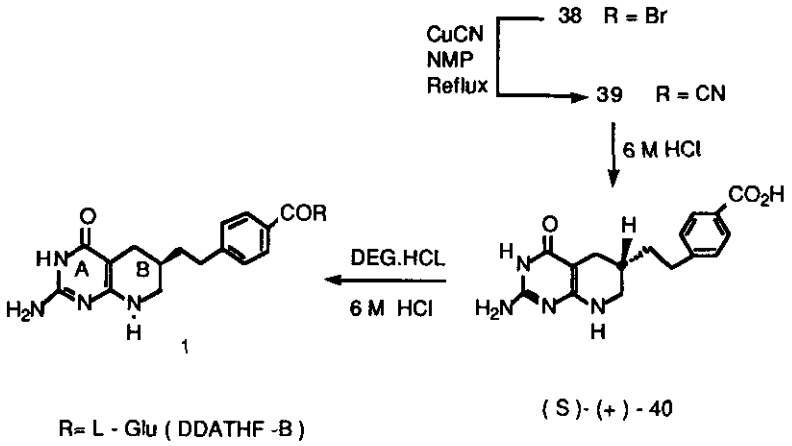
Scheme V.

Coupling of *tert*-butyl 4-ethenylbenzoate with methyl 2-chloro- 5-iodo-3-pyridinecarboxylate in the presence of palladium chloride and cuprous iodide lead to **26**. Cyclization with guanidine gave **27** and *tert*-butyl group

was removed to give **28**. Pivaloylation of **28**, followed by peptide coupling which was achieved in *N*-methylpyrrolidone as solvent with phenyl *N*-phenylphosphoramidochloridate as the coupling agent. DDATHF was prepared by reduction of **29** with Pd/C, followed by hydrolysis of the 2-pivaloyl and glutamate ester groups (Scheme V).

Barnett *et al.*[30] have reported the asymmetric synthesis of DDATHF **1** and related 5,10-dideaza-5,6,7,8-tetrahydropteroic acids. They have utilized enzymatic enantiodifferentiation of prochiral 1,3-diol **31** in the process (Scheme VI).[31] Reaction of **31** with MeOAc in the presence of porcine pancreatic lipase PPL gave the monoacetate (R)-(+)-**32**, 85%ee.[32] Mesylation of (R)-(+)-**32**, treatment with NaN_3 and hydrolysis produced azido alcohol (R)-(+)-**35**. Azido alcohol (R)-(+)-**35** was converted to **36** by tosylation and reaction with sodium diethyl malonate. Reduction of **36** gave **37**, reaction with the Meerwein reagent and exposure of the resulting lactim ether to guanidine produced **38**.[33] Reaction of **38** with CuCN afforded nitrile **39**.



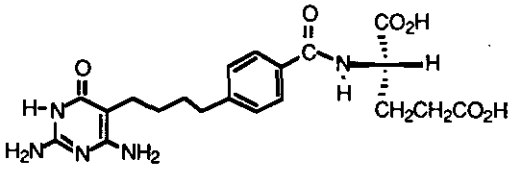


Scheme VI.

Hydrolysis of **39** gave (S)-(+)-5,10-didaza-5,6,7,8-tetrahidropteroic acid **40**. Chlorodimethoxytriazine was efficient and selective reagent for coupling of dideazatetrahydropteroic acid **40** with diethyl L-glutamate.[34] The asymmetric synthesis of Barnett *et al.* was significant, because it led to a single diastereomer. It also provided proof of the configuration of the two diastereomers and established the configuration relavite to natural tetrahydrofolate of the isomer selected for clinical trial.

The large scale fractional crystallization process was applied to separate the DDATHF diastereomers. However, this separation process appeared to be inapplicable to other DDATHF analogs.

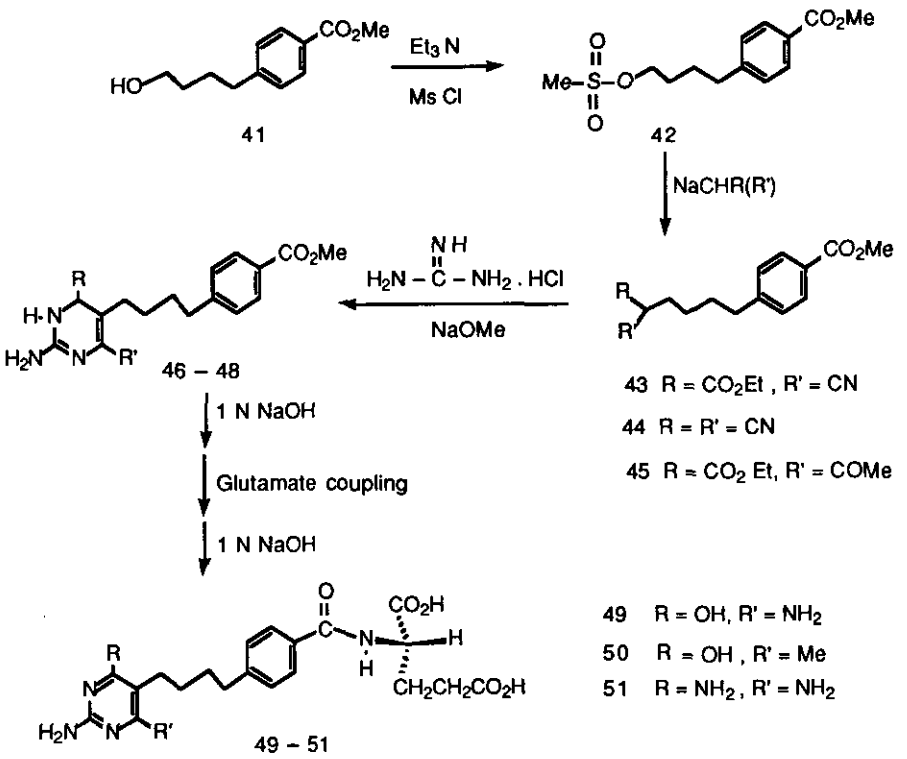
It is required to synthesis of DDATHF analogs possessing similar biological activities but lacking the asymmetric center at position 6. Removal of the methylene group at position 7 in DDATHF removes the chiral center at position 6 and gives an "open-chain" or des-methylene analogue (7-DM-DDATHF) **51** as a single enantiomer.(Figure II.)



51 7 - DM -DDATHF

Figure II.

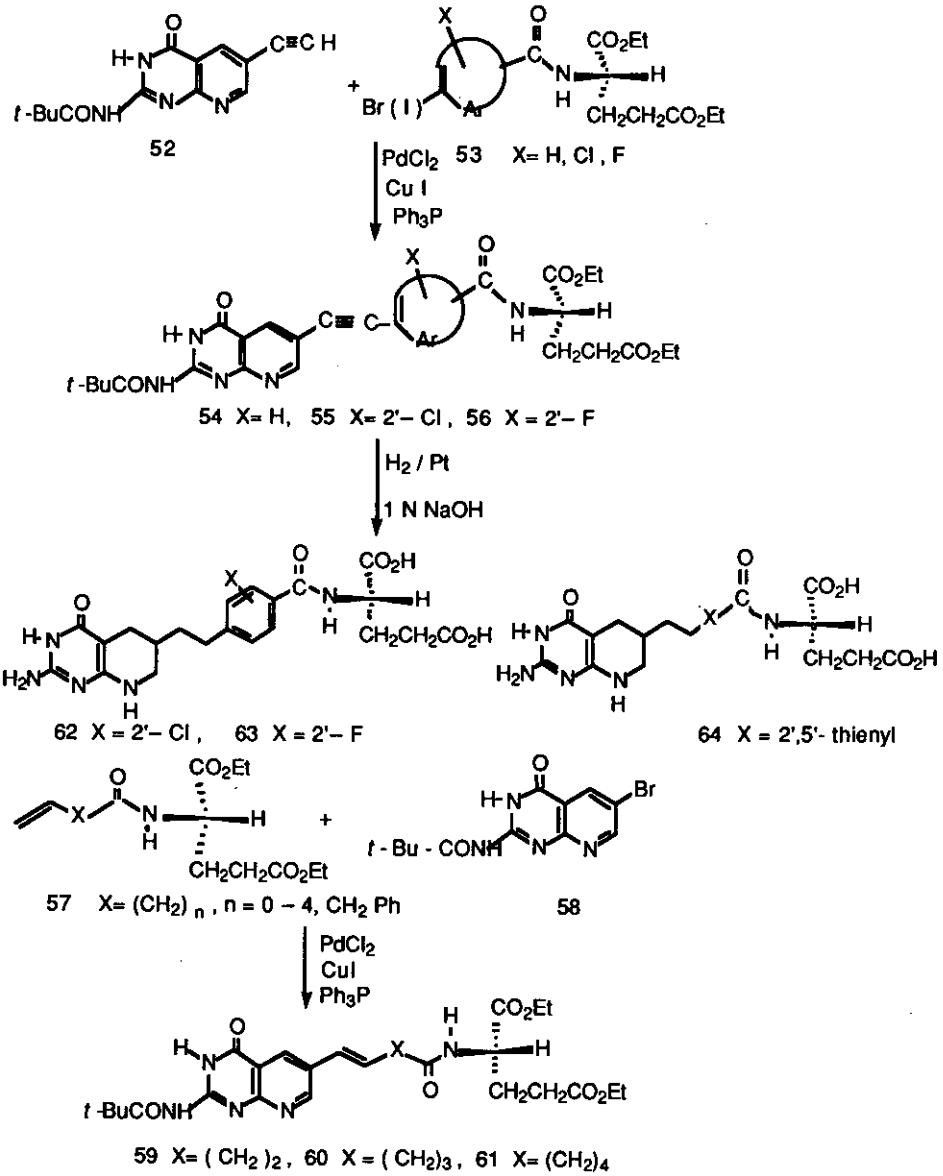
As part of their continuing structure-activity studies on DDATHF, Taylor and Harrington have achieved the synthesis of (7-DM-DDATHF) and its analogs **50-52** by the synthetic pathway [35] as shown in Scheme VII.

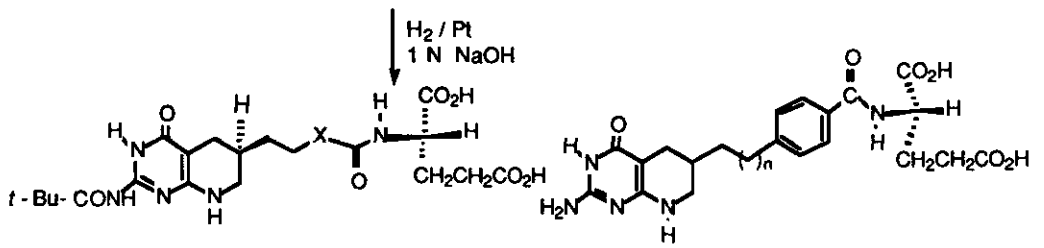


Scheme VII.

Mesylate was prepared from **41** and mesyl chloride, triethylamine. Alkylation of the anions of ethyl cyanoacetate, malononitrile and ethylacetoacetate with **42** gave compounds **43-45.** Guanidine cyclization was accomplished by using salt-free guanidine and adding it to compounds **43-45** in dimethylformamide. Hydrolysis of methyl esters **46-48** with NaOH followed by acidification with CH_3COOH afforded the free acids.Then the L-glutamate moiety was introduced using phenyl *N*-phenylphosphoramidochloridate as the coupling agent in N-methylpyrrolidone. Final saponification with NaOH followed by acidification [13] and desired products **49-51** was obtained. By using this strategy, very active DDATHF analogs have been prepared.

Construction of most the DDATHF analogues with variations in the phenyl region was achieved with the palladium catalyzed coupling reaction by Taylor's group[28,36] (Heck reaction).Compounds **54-56** were obtained by Heck reaction between 2-pivaloyl-6-ethynyl-5-deazapterin **52** and appropriately functionalized diethyl - iodo - (bromo) -aryloyl - L - glutamate **53.**In a similar way,coupling reaction of diethyl 4-ethynylbenzoylglutamate **57** with 2-pivaloyl-6-bromo-5-deazapterin **58** afforded **59-61** (Scheme VIII).

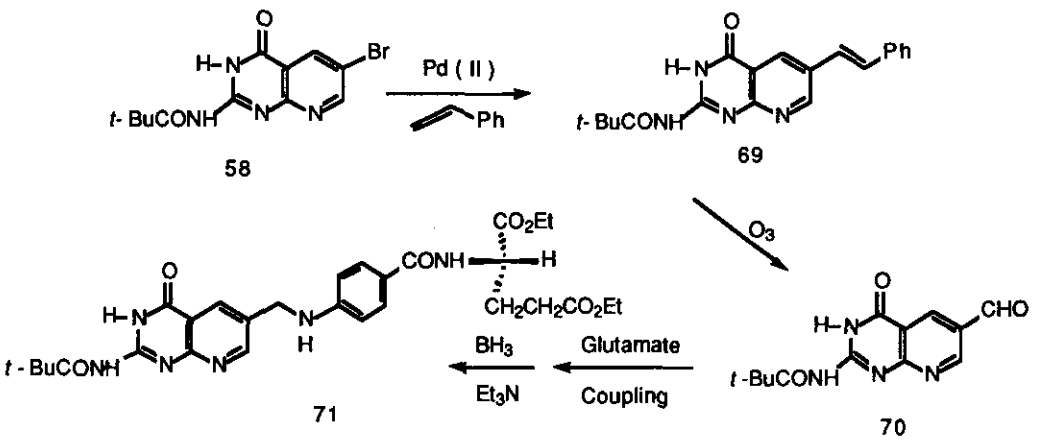

52
53 X= H, Cl, F
PdCl2
Cu I
Ph3P
54 X= H, 55 X= 2'– Cl, 56 X = 2'– F
H2 / Pt
1 N NaOH
62 X = 2'– Cl, 63 X = 2'– F
64 X = 2',5'- thienyl
57 X= (CH2)n, n = 0 – 4, CH2 Ph
58
PdCl2
CuI
Ph3P
59 X= (CH2)2, 60 X = (CH2)3, 61 X= (CH2)4

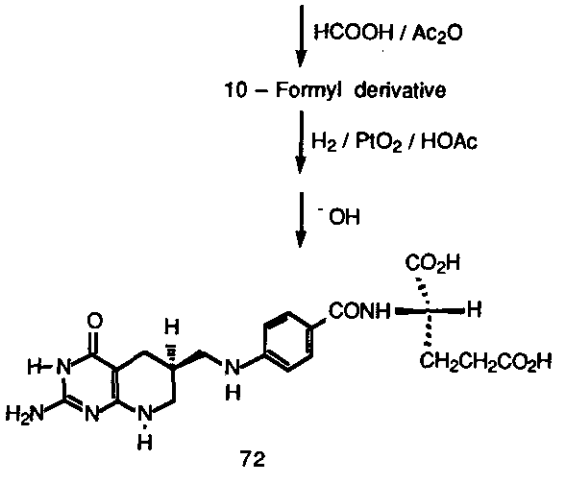


Scheme VIII.

Catalytic hydrogenation of intermediates **54-56** and **59-61**, followed by saponification with 1 N NaOH gave the final desired products **62-64** and **65-67**, homo DDATHF **68**.They also reported the biological activites of the DDATHF analogues modificated in the L-glutamate,benzene, 9,10-bridge and tetrahydropyridine region of the molecule .[37]

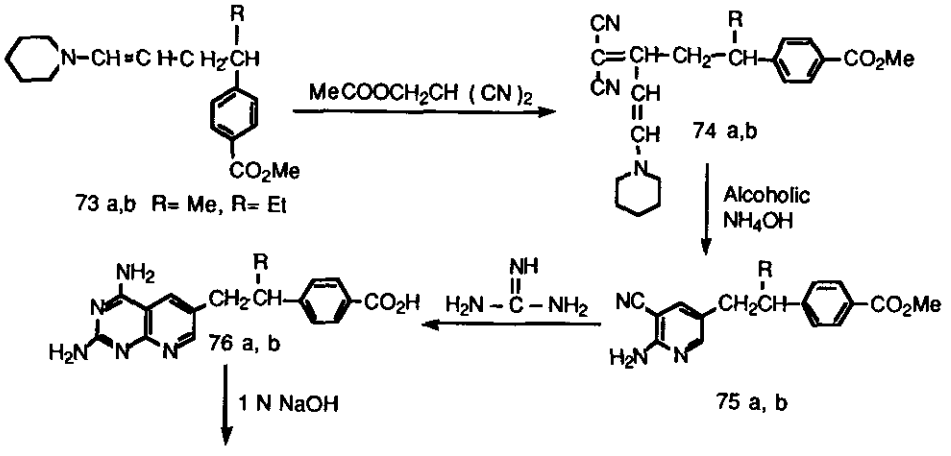
Taylor and Hamby have reported the synthesis of 5-deazatetrahydrofolic acid 5-DATHF .[13] They have used 2-pivaloylamino-6-bromo-5-deazapterin **58**, as the starting material (Scheme IX). By the Heck reaction, they have prepared 2-pivaloylamino-6-styryl-5-deazapterin **69**.[29] Ozonolysis of compound **69** afforded 2-*pivaloylamino-6-formyl-5-deazapterin* **70**, *glutamate coupling and reduction with* BH_3 gave diethyl 2-pivaloylamino-5-deazafolate **71**. 10-Formyl derivative of compound **71** was obtained by means of $HCOOH/Ac_2O$ mixture. Hydrogenation on PtO_2 in acidic media, and saponification with NaOH afforded the target compound **72**. This compound was found at least as active as DDATHF, both *in vitro* and *in vivo*. This result showed that substitution of nitrogen for carbon at position 10 of DDATHF was not detrimental either to its overall biochemical properties and to its antitumor activity.

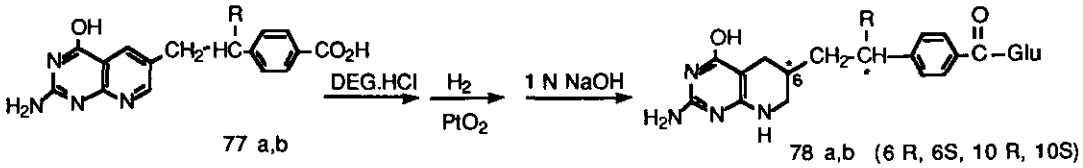




Scheme IX.

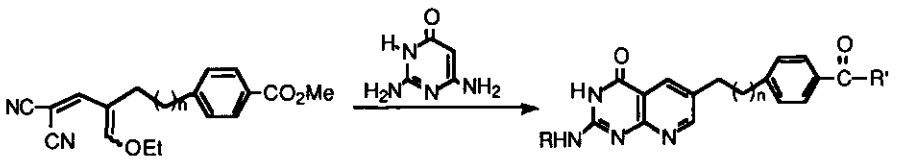
De Graw *et al.*[38] have reported the synthesis and activity of 10-methyl and 10-ethyl analogues of 5,10-dideazaminopterin. Key intermediates in the process (Scheme X) were 10-methyl- and 10-ethyl-4-amino-4-deoxy-5,10-dideazapteroic acids **76 a,b.**Condensation of the piperidine enamine of branched 4-(*p*-carbomethoxyphenyl)butyraldehydes **73a,b** with acetoxymethylenemalononitrile[39,40] afforded 1,1-dicyano-4-piperidinobutadiene **74 a,b.** Reaction of **74** with alcoholic NH_4OH gave substituted 2-amino-3-cyanopyridines **75 a,b.** Ring closure with guanidine yielded 10-methyl- and 10-ethyl-4-amino-4-deoxy - 5,10- dideazapteroic acids **76 a,b.** Hydrolysis of 4-amino group of **76 a,b** gave 10-alkylpteroic acids. Coupling with diethyl L-glutamate, hydrogenation on PtO_2 and saponification produced 10-alkyl-5,10-dideazatetrahydrofolic acids **78 a,b.**[26]





Scheme X.

Taylor and Harrington[41] have synthesized 5,10-dideaza-5,6,7,8-tetrahydrofolic acid **1** and its homolog 5,10-dideaza-5,6,7,8-tetrahydrohomofolic acid (HDDATHF) **68** by a new convergent strategy (Scheme XI).A key feature of the synthesis is the activation of carbonyl groups by aldol condensation with malononitrile. Condensation of **79** as a malondialdehyde equivalent[42] with 2,4-diamino-6(1*H*)-pyrimidone might be expected to take place more readily than with the elusive malondialdehyde itself. The Michael condensation of **79** with 2,6-diamino-4(1*H*)-pyrimidone led to **80.** Heating of **80** with 1 N NaOH resulted in hydrolysis of methyl ester. The resulting free acid **81a** was heated in refluxing pivalic anhydride in the presence of 4-(dimethylamino)pyridine as catalyst. The 2-pivaloylated mixed anhyride **82a** was obtained . Stirring of **82a** with 0.1 N NaOH gave the free acid **83a**,which, because of the 2- pivaloyl grouping was sufficiently soluble for further functionalization.[29] The glutamate side chain was introduced by using phenyl N-phenylphosphoramidochloridate as the coupling reagent in N-methylpyrrolidinone as solvent, and the resulting compound **84a** was reduced catalytically to the tetrahydroderivative **30.** DDATHF was prepared from this intermediate by hydrolysis of the ester functions and the pivaloyl protecting group.[28] Every step in the below ring construction reaction is facilitated by the greater electrophilicity of $C=C(CN)_2$ group as compared with carbonyl group, and by the fact that malononitrile anion is better leaving group than hydroxide ion.



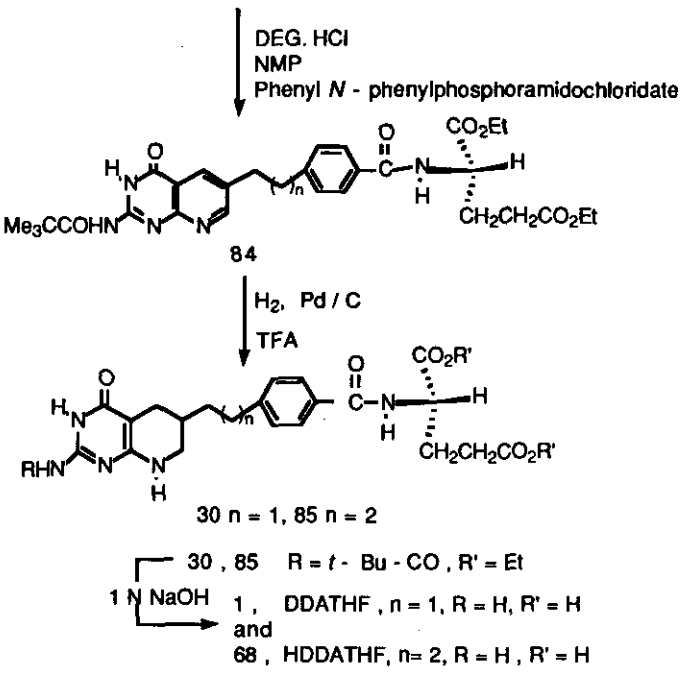
79 a, n = 1
b, n = 2

80

80 R= H, R'= OMe
1 N Na OH
81 R= H ,R '= OH

81
(*t* - Bu- $CO)_2O$
4 - DMAP
82 R = *t* - Bu-CO ,R' = *t* - Bu CO

82
0.1 N Na OH
83 R = *t* - Bu-CO , R = OH



Scheme XI .

Introduction of carbon substituents at C-10 of DDATHF like compound **86** (Figure III) leads to important increases in activity, possibly these such analogs resemble more closely than DDATHF itself the natural cofactor for GARFT (10-formyl- 5,6,7,8-tetrahydrofolic acid). In fact, the 10-hydroxymethyl derivative of DDATHF is known as the most potent DDATHF analog yet. This analog consists of a mixture of four diastereomers. [43] Taylor's group have separated these diastereomers on hplc cyclobond column, they have found only one of these as active.

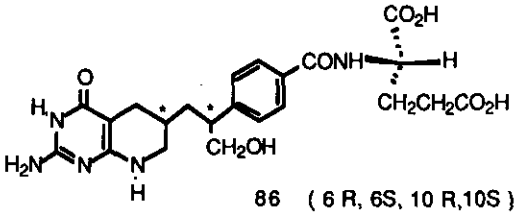
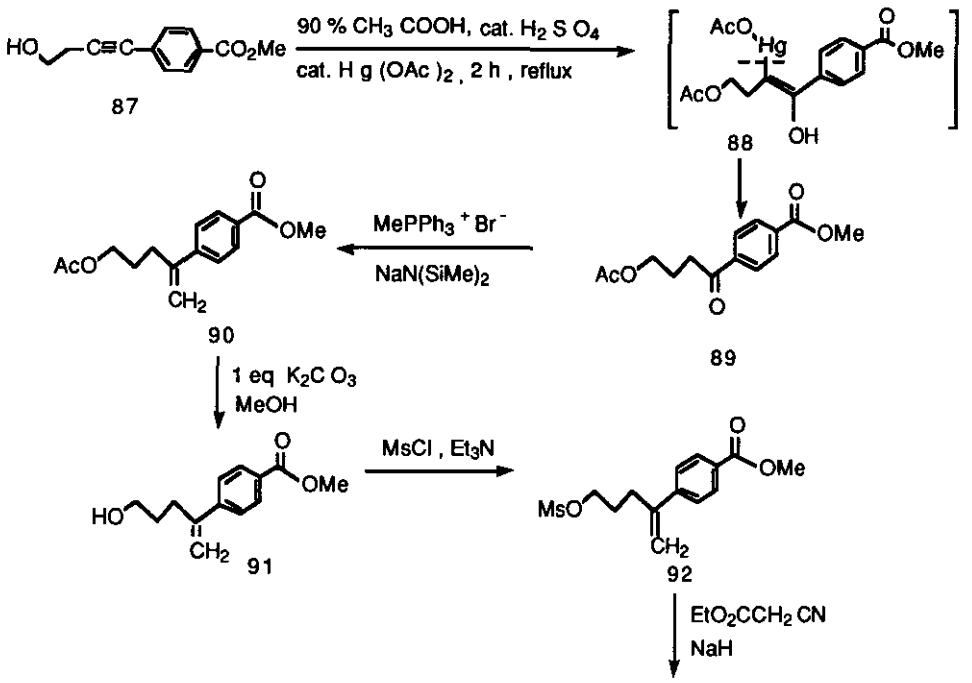


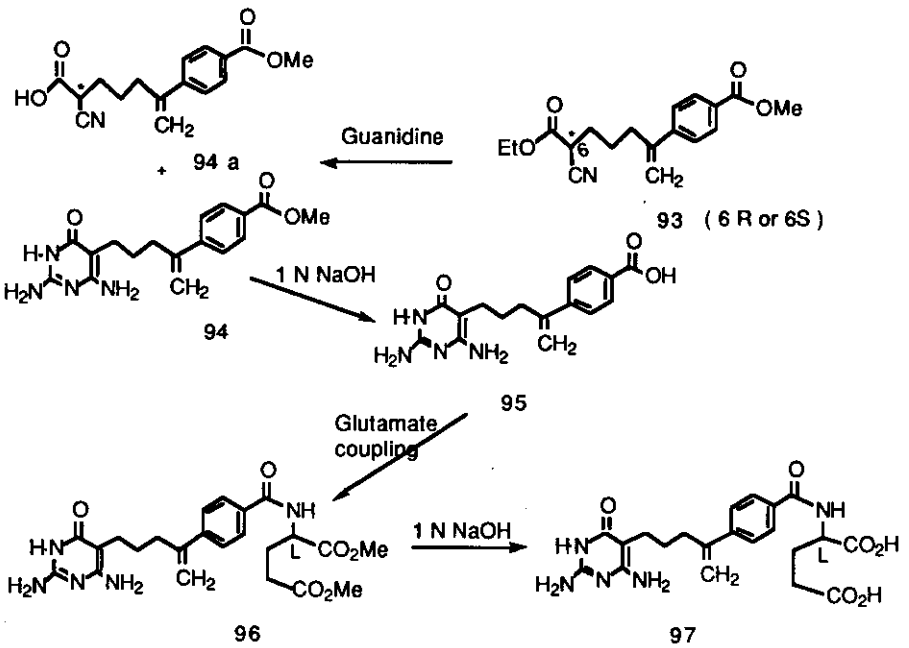
Figure III.

Their structure - activity studies have led to the synthesis of the "open - chain" analog of DDATHF **51** in which the C-7 methylene carbon have been deleted,[35] in this case C-6 chirality eliminates, but complete cytotoxicity almost retains.

Taylor's group approached the synthesis of the potential precursor **95** as shown in Scheme XII.[44] Palladium catalyzed coupling of 3-butyn -1-ol with methyl 4-bromobenzoate gave the alcohol **87**, which underwent

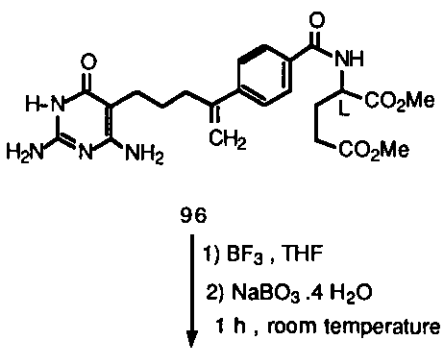
mercury-catalyzed hydration of the triple bond [45] to furnish **88**. Then mercury-carbon cleavage gave the aralkyl ketone **89**. Conversion of **89** to the methenyl derivative **90** was achieved by means of the subsequent Wittig olefination reaction. In this reaction , methyltriphenylphosphonium bromide and sodium hexamethyldisilazide in anhydrous THF[46] were used .By using potassium carbonate in methanol, *O*-acetyl group in **90** was selectively removed without cleavage of the methyl ester (Rapoport's deacetylation reaction).[47] The terminal hydroxyl group of **91** was converted to its mesylate **92**. Alkylation of the sodium salt of ethyl cyanoacetate with **92** gave **93**. Then cyclization with the free base of guanidine in refluxing methanol produced the desired pyrimidine intermediate **94**, along with some deesterified starting material **94a**. Alkaline cleavage of the benzoate, followed by glutamate coupling of the resulting carboxylic acid **95** using 2,4-dimethoxy-6-chloro-1,3,5-triazine / *N*-methylmorpholine as the coupling agent [34] gave **96**. This compound is a versatile precursor for a number of target 10-substituted "open-chain"·DDATHF analogs. Hydrolysis of **96** with aqueous NaOH followed by acidification yielded the glutamic acid derivative **97**. The 10-methenyl "open-chain" analog **97** was obtained in only of 8.0% yield on 11 steps.

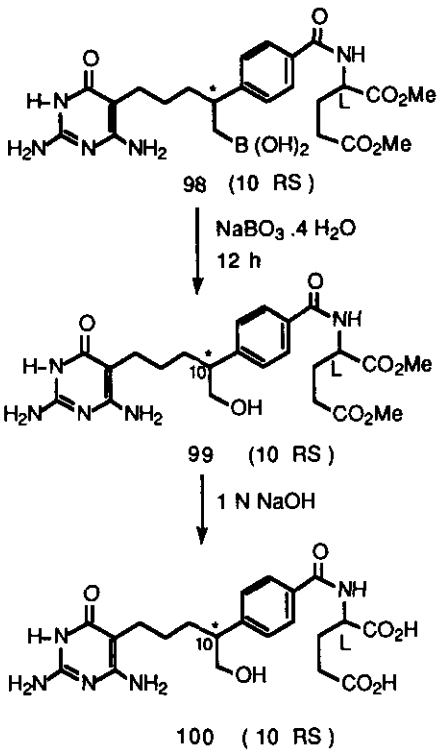




Scheme XII.

The 10-hydroxymethyl derivative of "open-chain" DDATHF analog was prepared from **96** by means of hydroboration reaction (Scheme XIII).[44] Interruption of the hydroboration-perboration reaction one hour after addition of $NaBO_3$ gave a stable intermediate **98** which is assumed as boronic acid.[48] Compound **98** was completely converted to 10-hydroxymethyl precursor **99** after continuining stirring with $NaBO_3$. Saponification of **99** with 1 N NaOH gave the glutamic acid **100.**





Scheme XIII.

The 10-methyl derivative **101** (Figure IV) was prepared by hydrogenation of **96** at 50 psi of hydrogen[44] in the presence of Pd/C as catalyst. After this reduction, glutamate was obtained,thereafter,the ester was hydrolyzed with NaOH to afford 10-methyl "open-chain" analog **101**.

These open-chain DDATHF analogs are inactive against TS and DHFR, inhibit purine de novo biosynthesis, as similar to DDATHF itself.

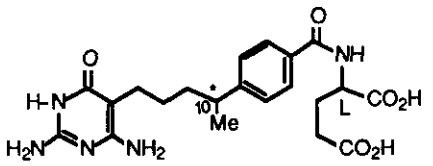


Figure IV.

In brief, DDATHF was designed as an inhibitor of folate metabolism, at a locus other than DHFR. The absence of nitrogen atoms at positions 5-and 10-prevents DDATHF serving as a cofactor in any of the one-carbon transfers of folate metabolism. The presence of a 2-amino-4-oxopyrimidine[9] "A" ring and a fully reduced "B" ring would make DDATHF unlikely to be an inhibitor of DHFR. The presence of a normal *p*-

benzoylglutamete moiety would allow conversion to polyglutamate forms. The presence of carbon rather than nitrogen at position 5 avoids the chemical instability which otherwise plagues tetrahydrofolates. All of the predictions based on its structure have been found to be correct. DDATHF has proven to be a potent anti-tumor agent *in vivo* and *in vitro*. The studies of the reversal of cyctotoxicity by various metabolites suggested that the site of DDATHF action lies in *de novo* purine biosynthesis. DDATHF produced a nearly 10 fold decline in ATP and GTP with a concentration dependence very similar to that seen for cyctotoxicity.

Previus studios with DDATHF indicated that parent or a polyglutamyl derivative was a potent inhibitor of *de novo* purine nucleotide biosynthesis.[9,12] Since the cyctotoxicity of DDATHF in cultured cell appears to dependent on the depletion of intracellular purine nucleotide levels, it was of interest to study the effects of DDATHF on the growth and differentitation of HL-60 promyelocytic leukemia cells. Galivan *et al.* described that different concentrations of lipid -soluble DHFR inhibitors and MTX could cause synergistic growth inhibition and cell kill of hepatoma cells in vitro, when coupled with PDDF.[49] Their results suggested that the DHFR inhibitors act by increasing the capaticy of PDDF to inhibit TS.[50] Sokoloski *et al.*[51] demonstrated that DDATHF was a potent inducer of maturation of HL-60 leukemia cells. The induction of differentitation by DDATHF was associated with the inhibition of *de novo* purine nucleotide biosynthesis, presumably at the reaction catalyzed by GARFT.[9,12,28] These findings efforts the importance of purine nucleotides to both the growth and differentiation of HL-60 leukemia cells .[9,24]

DDATHF has no inhibitor activity against either DHFR or TS in vitro. But it was found to be an excellent substrate [12] for FPGS and to deplete cellular ATP and GTP at concentrations in the range of 10-30 nm as inhibitor to leukemic cell growth.[52,53] The 6S-and 6R-diastereomers of DDATHF are moderately inhibitors of 5'-phosphoribosylglycinamide formyl transferase.[54,55] The two diastereomers were also efficient substrates for mouse liver FPGS.[11,56] Pizzorno *et al.*[57] have reported that (6R)-DDATHF was rapidly converted polyglutamates in the cultured human leukemia cell lines. Polyglutamylation of (6R)-DDATHF represented a mechanism for trapping the drug inside the cells producing a more potent inhibitor of the target enzyme.

3. CONCLUSION

DDATHF was found as a chemotherapeutic agent against experimental rodent solid tumors [9,58] and also, during its first use in patients with cancer.[59] Moran *et al.*[60] have compared the activity of a series of DDATHF analogs as inhibitors of GARFT purified to electrophoretic homogeneity from mouse L 1210 cells. They indicated that a reduced pyridopyrimidine ring, N-8 and 2- amino group of DDATHF played an important role in the binding of tetrahydrofolate analogs to GARFT [56] and the glutamic acid in the side chain of DDATHF did not play a role in this ligand-enzyme interaction. Polyglutamates of DDATHF are much more active inhibitors of GARFT than is the parent molecule .[61] Activity of DDATHF analogs retained in

case of replacing phenyl ring by a cyclohexyl ring or by methylene groups.

The abbreviations used are: DHFR, dihydrofolate reductase; PDDF, N^{10}-propargyl-5,8-dideazafolate *N*{4-[*N*-(2-amino-4-hydroxy-6-quinazolinyl) methyl]prop-2-ynl amino}benzoyl-L-glutamic acid, AICAR,Amino-imidazolecarboxamideribotide; TS, Thymidylate synthetase; FPGS, Folypolyglutamate synthetase, ATP, Adenosine triphosphate; GTP, Glucose-3-phosphate.

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