with Et_2O until a precipitate formed. The solid (unreacted acetone semicarbazone) was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was added to 10% H_2SO_4 (500 ml). A solution of 5-nitro-2-furaldehyde (113 g, 0.8 mol) in absolute EtOH (500 ml) was added and the mixture was heated on the steam bath for 1 hr. The solution was chilled and filtered to yield 51 g. The product was recrystallized from $MeNO_2$ (charcoal).

1-[(5-Nitrofurfurylidene)amino]-4-(4-nitrophenyl)-4-imidazolin-2-one (4). To an aqueous MeOH solution of 5-nitro-2-furaldehyde (28.2 g, 0.20 mol) was added 1-acetamido-4-(4-nitrophenyl)-4-imidazolin-2-one[3] (52.4 g, 0.20 mol). The mixture was acidified with concentrated HCl and heated on the steam bath for 2 hr. After chilling, the product was collected by filtration and dried to yield 58.0 g. The material was recrystallized from aqueous DMF (charcoal).

In a similar manner, **5** was prepared from 1-acetamido-4-(4-chlorophenyl)-4-imidazolin-2-one[3] and recrystallized from aqueous DMF.

4-Methyl-1-[(5-nitrofurfurylidene)amino]-4-imidazolin-2-one (7). To a solution of 5-nitro-2-furaldehyde 2-(hydroxypropyl)semicarbazone (**6**,[6] 37.0 g, 0.14 mol) in anhydrous DMSO (435 ml) was added Ac_2O (290 ml). The mixture was stirred at 50° for 18 hr and poured into a large volume of ice and water. The brick-red precipitate was collected, washed with H_2O, and dried to yield 26 g. The product was purified by recrystallization from $MeNO_2$ (charcoal).

In a similar manner, **9** was prepared from **8** and recrystallized from $MeNO_2$. Material recrystallized from $MeNO_2$ formed a complex which was decomposed by drying at 70° for 2 hr. Compound **8** was prepared by the method of Gever[7] and recrystallized from aqueous EtOH (charcoal): mp 184.5–186°. Anal. ($C_{10}H_{14}N_4O_5$) C, H, N. Compound **11** was synthesized in the above manner from **10**[7] and recrystallized from $MeNO_2$.

Acknowledgments. The authors gratefully acknowledge the aid of Mr. Frederick Abbott and Mr. Alexander Winterstein for the preparation of chemical intermediates. Mr. Grant Gustin and Mr. Marvin Tefft performed the microanalyses. The microbiologic tests were performed by Patrick Moynihan.

References and Notes

(1) D. L. Pugh, J. Olivard, H. R. Snyder, Jr., and J. P. Heotis, *J. Med. Chem.*, **15**, 270 (1972).
(2) U.S. Adopted Name (USAN) of 1-[(5-nitrofurfurylidene)-amino]-2-imidazolidinone.
(3) H. Beyer and A. Hetzheim, *Chem. Ber.*, **97**, 1031 (1964).
(4) J. D. Albright and L. Goldman, *J. Am. Chem. Soc.*, **87**, 4214 (1965).
(5) R. Freedman and R. E. Chamberlain, *Antimicrob. Agents Chemother.*, 502 (1967).
(6) G. Gever, C. O'Keefe, G. Drake, F. Ebetino, J. Michels, and K. Hayes, *J. Am. Chem. Soc.*, **77**, 2277 (1955).
(7) G. Gever, *J. Am. Chem. Soc.*, **76**, 1283 (1954).

Synthesis of a Fluorescent Derivative of Amethopterin[1a]

G. R. Gapski, J. M. Whiteley, J. I. Rader,[1b] P. L. Cramer, G. B. Henderson,[1c] V. Neef, and F. M. Huennekens*

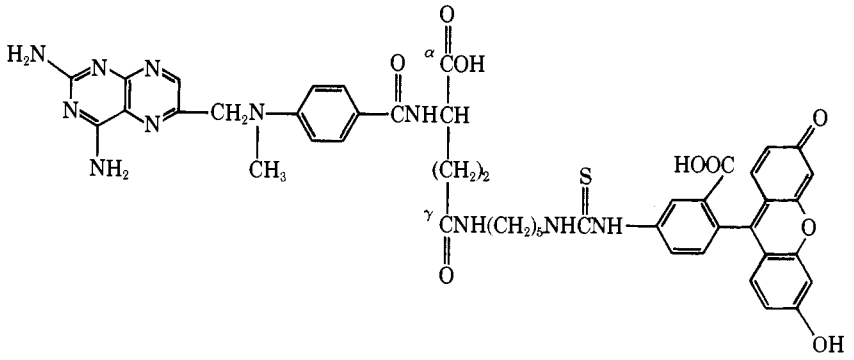
Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received November 25, 1974*

Fluorescein isothiocyanate was treated with excess diaminopentane and the remaining unsubstituted amino group of the product was condensed, via a carbodiimide-promoted reaction, with a carboxyl group of amethopterin. The final product, a fluorescent derivative of amethopterin, was isolated by chromatography on AE-cellulose and preparative electrophoresis on polyacrylamide. It was shown to be homogeneous by analytical polyacrylamide electrophoresis and thin-layer chromatography. Proof of structure was provided by elemental analysis, absorbance spectra (at pH 7.0, λ_{max} at 495 nm; fluorescence emission at 520 nm), and ^1H NMR measurements. The fluorescent derivative of amethopterin inhibited transport of amethopterin into *Lactobacillus casei* and L1210 cells. It was also a good inhibitor of the *L. casei* and L1210 dihydrofolate reductases and could be used to provide a fluorescent label for the enzymes during polyacrylamide electrophoresis.

Amethopterin (Methotrexate, MTX) is a potent inhibitor of dihydrofolate reductases from a variety of sources.[2] Although the affinity of the enzymes for the inhibitor is quite high, the interaction is noncovalent and pH-dependent.[3] These properties have been exploited in numerous studies in which radioactive MTX has been used to detect and quantitate the enzyme. We now wish to report the synthesis of a fluorescein derivative of MTX (MTX-F) which can be used as a *visual* label for dihydrofolate reductases and for MTX-transport systems.

Derivatization of MTX was accomplished via one of the terminal carboxyl groups, since this region of the molecule is minimally involved in binding to dihydrofolate reductases. This is shown by the fact that various esters and amides of MTX are only slightly less inhibitory than the parent compound[4,5] and by the successful use of MTX linked through its carboxyl groups to soluble starch[6] or Sepharose[7-9] in the purification of these enzymes by affinity chromatography. Preparation of MTX-F was carried out in two steps. Initially, fluorescein isothiocyanate was treated with excess of 1,5-diaminopentane in dimethyl sulfoxide and the resulting thiourea was purified by chromatography on DEAE-cellulose. Homogeneity was confirmed by thin-layer chromatography (TLC) on cellulose in two solvent systems, and the presence of the remaining amino group was demonstrated by reactivity with ninhydrin. The infrared spectrum indicated a loss of the characteristic isothiocyanate peak at 2100 cm^{-1}. In the second step, MTX was allowed to react with 1 equiv of the preceding product in the presence of carbodiimide. The reaction mixture was chromatographed on AE-cellulose and elution was achieved with a linear gradient of ammonium bicarbonate. The major component was purified further by preparative polyacrylamide electrophoresis and rechromatography on AE-cellulose. Acid precipitation afforded the desired product (MTX-F) as a yellow, amorphous solid. The purity and authenticity of MTX-F was confirmed by the absorbance spectrum, TLC, elemental analysis, and the ^1H NMR spectrum (which indicated a 1:1 superimposition of the aminoalkylfluorescein aromatic proton resonances on the aromatic side-chain resonances of MTX in the region 7–8 ppm).

From the method of preparation and analytical data, it is concluded that the diaminopentane spacer group is linked covalently to either the α- or γ-carboxyl group of MTX. The natural occurrence of γ-linked folate polyglutamates (reviewed by Wagner and Folkers[10]) and the large size of



1

Table I. Inhibition of Dihydrofolate Reductases and MTX Transport by MTX-F

System[a]	Source L1210	Source *L. casei*
Dihydrofolate reductase		
K_i, MTX-F	62 n*M*	45 n*M*
K_i, MTX	41 n*M*	15 n*M*
MTX transport		
K_i, MTX-F	0.5 μM	1.6 μM
K_m, MTX	0.35 μM	1.3 μM

[a] Assay conditions are defined in the Experimental Section.

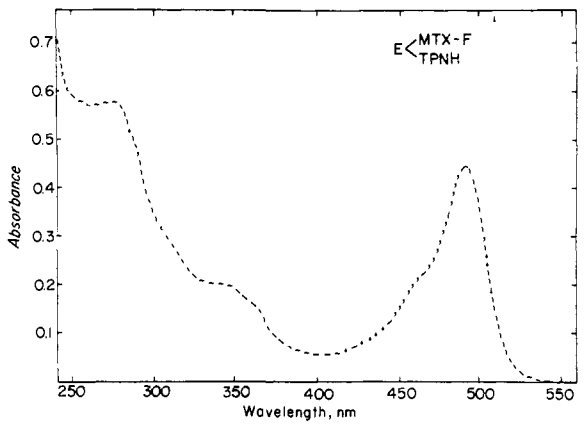


Figure 1. Absorbance spectrum of the ternary complex between L1210 dihydrofolate reductase, MTX-F, and TPNH. Pure L1210 dihydrofolate reductase (8.8 μM) was admixed with a threefold molar excess each of MTX-F and TPNH in 0.1 *M* phosphate buffer, pH 7.0, and the solution was dialyzed exhaustively against the same buffer.

the fluorescent component may indicate that formation of the γ-substituted MTX-F (**1**) would be favored in this case.

The absorbance spectrum of MTX-F was a composite of the two chromophoric constituents. Its most prominent features were the long-wavelength absorbance of MTX at 370 nm and the characteristic fluorescein peak at 495 nm. At pH 7.0, the millimolar extinction coefficient for the latter was 55.3. Solutions of MTX-F (pH 7.0), when irradiated at 493 nm, showed a fluorescence maximum at 520 nm with a quantum yield (QY) of 0.22.

Introduction of the fluorescein moiety at the carboxyl end of MTX did not appreciably diminish the strong interaction with dihydrofolate reductases (Table I). MTX-F also possessed a good affinity for MTX transport systems, as shown by its ability to inhibit uptake of [^{3}H]-MTX by L1210 and *L. casei* cells (Table I). In the latter experiments, the K_i values for MTX-F were very similar to the K_m values for the transport of MTX. The affinity of MTX and TPNH for the *L. casei* and L1210 dihydrofolate reductases has allowed various binary and ternary complexes to be separated by electrophoresis on polyacrylamide gels; previously, bands have been located[11-13] by staining for protein or enzymatic activity. By replacing MTX with MTX-F, these complexes can be seen, without stains, as bright yellow-green fluorescent bands when the gels are examined under ultraviolet light. However, in contrast to the single electrophoretic bands which result from interaction of MTX and TPNH with the enzymes, MTX-F under the same conditions gives rise to multiple bands. This may be due to isomeric forms of the MTX-F (see above) whose subtle differences are, nevertheless, recognizable by the enzyme. These results indicate that MTX-F can be used to mark dihydrofolate reductases during purification or other manipulations. The enzymes can also be quantitated via complex formation with MTX-F (in the presence of TPNH), as illustrated in Figure 1. After exhaustive dialysis, the concentration of the bound MTX-F was calculated from the absorbance at 495 nm (corrected for the small change in ϵ that occurs when the chromophore is associated with enzyme) and shown to be equimolar with enzyme. The success of this method rests upon the fact that the maximum absorbance of the fluorescein moiety occurs at a wavelength where few other biological compounds absorb. Changes in fluorescence of the chromophore, which have been observed when MTX-F interacts with the enzyme under various conditions, suggest the feasibility of using this compound as a probe for the dihydrofolate/MTX binding site.

Experimental Section

Fluorescein-Diaminopentane. Fluorescein isothiocyanate (600 mg, 1.54 mmol) was treated with 1,5-diaminopentane (1.57 g, 15.4 mmol) in dimethyl sulfoxide (8 ml) for 4 hr at room temperature. The product was purified by chromatography on DEAE-cellulose using 0.3 *M* NH_4HCO_3 buffer, pH 7.8, as the eluent. Lyophilization followed by acid precipitation from a dilute ammonium hydroxide solution gave the desired product as an amorphous yellow solid (590 mg, 78%): TLC on cellulose, R_f 0.82 in MeOH–NH_4OH–H_2O (7:1:2) and 0.29 in 0.1 *M* potassium phosphate, pH 7; absorbance max (0.1 *M* K phosphate, pH 7) 493 nm (ϵ 67,200); absorbance max (0.1 *M* NaOH) 487 nm (ϵ 79,800); fluorescence max (0.1 *M* K phosphate, pH 7) excitation, 493 nm; emission, 525 nm (QY = 0.53); ^{1}H NMR (trifluoroacetic acid-d_1) δ 1.9 (m), 3.3 (m), and 3.7 (m) (10 aliphatic protons), 7.4 (m), 7.9 (d), and 8.5 (s) (9 aromatic protons). Anal. ($C_{26}H_{25}N_3O_5S \cdot 2H_2O$) C, H, N.

Fluorescein-Diaminopentane-Amethopterin (MTX-F). Fluorescein-diaminopentane (491 mg, 1 mmol) was allowed to react with MTX (454 mg, 1 mmol) in the presence of excess 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (1.00 g, 5.22 mmol) in dimethyl sulfoxide (15 ml) for 1 hr at room temperature. The preparation was chromatographed on a column of AE-cellulose using a linear gradient of 0.1→0.5 *M* NH_4HCO_3 as the eluent. A single broad band was observed in the elution profile; peak frac-

tions were pooled and lyophilized. The residue (in 100-mg batches) was dissolved in 0.1 M NH_4OH, and the resulting solutions were subjected to preparative polyacrylamide gel electrophoresis using 0.1 M Tris–chloride buffer, pH 8.1, as the eluent. After rechromatography on AE-cellulose, the product (MTX-F) was again lyophilized and then further purified by acid precipitation from 0.1 M NH_4OH (178 mg, 18%): TLC on cellulose, R_f 0.71 in MeOH–NH_4OH–H_2O (7:1:2) and 0.67 in 0.25 M NH_4HCO_3 saturated with n-BuOH; absorbance max (0.1 M K phosphate, pH 7) 258 nm (ϵ 48,200), 365 (12,600), 495 (55,300); absorbance max (0.1 M NaOH) 284 nm (ϵ 36,400), 375 (12,600), 493 (67,400); fluorescence max (0.1 M K phosphate pH 7) excitation, 493 nm; emission, 520 nm (QY = 0.22); ^1H NMR (trifluoroacetic acid-d_1) δ 7.42 (m) and 8.46 (m) (9 H, fluorescein aromatic), 7.92 (m, 4 H, MTX aromatic), and 8.82 (s, 1 H, C-7). Anal. ($C_{46}H_{45}N_{11}O_9S \cdot 3.5H_2O$) C, H, N.

Materials and Methods. Fluorescein isothiocyanate (isomer 1) and diaminopentane were obtained from Sigma Chemical Co. MTX was a gift from Dr. Florence White, National Cancer Institute; [^3H]-MTX was supplied by Dhom Products Ltd. Absorption, fluorescence, infrared, and ^1H NMR spectra were taken with Cary Model 14, Turner Model 210, Perkin-Elmer Model 337, and Jeol Model JNM-PS-100 (courtesy of Dr. J. Rivier, Salk Institute for Biological Studies) spectrometers, respectively. Quantitative polyacrylamide electrophoresis employed a Buchler Poly-Prep 200 instrument.

Dihydrofolate reductases were assayed spectrophotometrically.[14] Transport of [^3H]-MTX into *L. casei*[15] and L1210[16] cells was measured according to the indicated procedures. Enzymatic and transport inhibition constants were obtained from plots of $1/v$ against i at varying substrate concentrations where v is the initial rate of reaction or transport and i is the inhibitor concentration.

Disk electrophoresis on 7.5% (w/v) polyacrylamide gels was carried out in 0.6 × 6 cm tubes. Protein and enzymatic activity were visualized by the staining procedures of Dunlap et al.[17]

Acknowledgments. The authors are indebted to Dr. U. J. Lewis for advice on preparative gel electrophoresis.

References and Notes

(1) (a) This work was supported by grants from the National Cancer Institute, National Institutes of Health (CA 6522 and CA 11778), and the American Cancer Society (BC-62K). (b) Postdoctoral Fellow of the American Cancer Society. (c) Recipient of a Junior Fellowship of the American Cancer Society, California Division.
(2) (a) F. M. Huennekens, R. B. Dunlap, J. H. Freisheim, L. E. Gundersen, N. G. L. Harding, S. A. Levison, and G. P. Mell, *Ann. N.Y. Acad. Sci.*, **186**, 85 (1971); (b) R. L. Blakley, "The Biochemistry of Folic Acid and Related Pteridines", North Holland Press, Amsterdam, 1969, Chapter 5.
(3) J. R. Bertino, B. A. Booth, A. L. Bieber, A. Cashmore, and A. C. Sartorelli, *J. Biol. Chem.*, **239**, 479 (1964).
(4) M. Chaykovsky, A. Rosowsky, N. Papathanasopoulos, K. N. Chen, E. J. Modest, R. L. Kisliuk, and Y. Gaumont, *J. Med. Chem.*, **17**, 1212 (1974).
(5) D. G. Johns, D. Farquhar, M. K. Wolpert, B. A. Chabner, and T. L. Loo, *Drug Metab. Dispos.*, **1**, 580 (1973).
(6) G. P. Mell, J. M. Whiteley, and F. M. Huennekens, *J. Biol. Chem.*, **243**, 6074 (1968).
(7) J. M. Whiteley, R. C. Jackson, G. P. Mell, J. H. Drais, and F. M. Huennekens, *Arch. Biochem. Biophys.*, **150**, 15 (1972).
(8) B. T. Kaufman and J. V. Pierce, *Biochem. Biophys. Res. Commun.*, **44**, 608 (1971).
(9) J. S. Erickson and C. K. Mathews, *Biochem. Biophys. Res. Commun.*, **43**, 1164 (1971).
(10) A. F. Wagner and K. Folkers, "Vitamins and Coenzymes", Interscience, New York, N.Y., 1964, Chapter 7.
(11) L. E. Gundersen, R. B. Dunlap, N. G. L. Harding, J. H. Freisheim, F. Ötting, and F. M. Huennekens, *Biochemistry*, **11**, 1018 (1972).
(12) F. Ötting and F. M. Huennekens, *Arch. Biochem. Biophys.*, **152**, 429 (1972).
(13) F. M. Huennekens, P. M. DiGirolamo, K. Fujii, G. B. Henderson, D. W. Jacobsen, V. G. Neef, and J. I. Rader, *Adv. Enzyme Regul.*, **12**, 131 (1974).
(14) R. C. Jackson and F. M. Huennekens, *Arch. Biochem. Biophys.*, **154**, 192 (1973).
(15) G. B. Henderson and F. M. Huennekens, *Arch. Biochem. Biophys.*, **164**, 722 (1974).
(16) J. I. Rader, D. Niethammer, and F. M. Huennekens, *Biochem. Pharmacol.*, **23**, 2057 (1974).
(17) R. B. Dunlap, L. E. Gundersen, and F. M. Huennekens, *Biochem. Biophys. Res. Commun.*, **42**, 772 (1971).

Inhibition of Phenylalanyl-tRNA Synthetase by Aromatic Guanidines and Amidines[1]

Peter V. Danenberg

Wisconsin Clinical Cancer Center, The Medical School, University of Wisconsin, Madison, Wisconsin 53706

and Daniel V. Santi*

Department of Pharmaceutical Chemistry and Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143. *Received November 15, 1974*

Aromatic guanidines and amidines were investigated for their ability to inhibit phenylalanyl-tRNA synthetase from *E. coli* B. 2-Phenylacetamidine (**1**), benzylguanidine (**2**), and *N*-benzylbenzamidine (**3**) are competitive inhibitors with respect to phenylalanine, binding nearly as well as the substrate. The remainder of the inhibitors was unexpectedly found to be noncompetitive, indicating the presence of a secondary binding site on the enzyme. Inhibition by these compounds appears to be specific for phenylalanyl-tRNA synthetase and requires the presence of a phenyl ring as well as the amidine or guanidine moiety.

The aminoacyl-tRNA synthetases are a class of ligating enzymes that activate amino acids and attach them to the 3′ terminus of their cognate tRNA's.[2a] Each of the naturally occurring amino acids is activated with specificity by its own synthetase.[2b]

One of our objectives in a broader study of these enzymes has been to elucidate the structural features of phenylalanine critical for its recognition by phenylalanyl-tRNA synthetase and to probe the topography and localized environment of the binding site of this enzyme. By measuring the binding of a large number of substrate analogs incorporating systematic structural changes, we have been able to demonstrate that the major requirements for binding and recognition of phenylalanine analogs by phenylalanyl-tRNA synthetase are an unsubstituted phenyl ring and a protonated amino group, separated by a distance equivalent to two methylene groups.[3] The carboxyl group does not appreciably contribute to binding, and a variety of groups can be substituted into this region.

A knowledge of these factors provides the basis for de-