The 6S- and 6R-Diastereomers of 5,10-Dideaza-5,6,7,8-tetrahydrofolate Are Equiactive Inhibitors of de Novo Purine Synthesis*

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The diastereomers of 5,10-dideaza-5,6,7,8-tetrahydrofolate (DDATHF) differing in chirality about carbon 6 were resolved and studied as inhibitors of folate-dependent processes in mouse leukemia cells. Both diastereomers of DDATHF were found to be potent inhibitors of leukemia cell growth due to effects on de novo purine synthesis. Cell growth inhibition by these compounds was prevented by 5-formyltetrahydrofolate in a dose-dependent manner. This indicated that the effects of the DDATHF diastereomers were due to inhibition of folate-dependent processes. No metabolite reversal experiments indicated that 5'-phosphoribosylglycinamide formyltransferase was the major site of action of these compounds in mouse cells. Another site in de novo purine synthesis was affected at higher concentrations of diastereomer B in L1210 cells. Low concentrations of both diastereomers were found to inhibit pure L1210 5'-phosphoribosylglycinamide formyltransferase competitively with the folate substrate. The two diastereomers were also efficient substrates for mouse liver folylypolyglutamate synthetase.

We conclude that the 6R- and 6S-diastereomers of DDATHF are remarkably similar and equiactive anti-metabolites inhibitory to de novo purine synthesis and that the biochemical processes involved in their cytoxicity display little stereochimical specificity.

The utility of the folate anti-metabolite methotrexate, a potent inhibitor of dihydrofolate reductase (EC 1.5.1.3), as a cancer chemotherapeutic agent and in the treatment of psoriasis has made it clear that antimetabolites of folate cofactor metabolites in which the lead compound was 5,10-dideaza-5,6,7,8-tetrahydrofolate (DDATHF). We have previously shown (2) that DDATHF inhibits de novo purine synthesis in cultured mouse and human leukemia cells and have presented indirect evidence that it does so by inhibiting 5'-phosphoribosylglycinamid formyltransferase (EC 2.1.2.2) in L1210 cells.

DDATHF, as originally reported, is a mixture of two diastereomers differing in chirality at carbon 6. It was not clear whether the biological/biochemical properties (1, 2) of DDATHF were caused by one of these chemically distinct compounds or whether the different activities of DDATHF were characteristics of the two different diastereomers. We have recently resolved the mixture of diastereomers of DDATHF into the 6R- and 6S-components of sufficient diastereomeric purity (>;97%) to allow these questions to be answered. We now report that the two diastereomers of DDATHF are both inhibitors of de novo purine synthesis equiactive with the parent mixture, that both compounds are potent competitive inhibitors of 5'-phosphoribosylglycinamide formyltransferase, and that the biochemical processes involved in the cytotoxicity of DDATHF show a remarkably low level of stereochimical specificity.

MATERIALS AND METHODS

The concentrations of stock solutions of DDATHF were spectrophotometrically determined using ε₅₅₀ values of 11,700 M⁻¹ cm⁻¹ (at 272 nm) in 0.1 N NaOH. 5,10-Dideaza-5,6,7,8-tetrahydropteroyl-L-aspartate (DDATH-PteAsp) was made by a modification of the procedure for the synthesis of DDATHF (1). (6R)-10-Formyltetrahydrofolate (10-CHO-H₄PteGlu) was prepared from (6S)-5-formyl-10-CHO-H₄PteGlu, 10-formyl-5,8-dideazapteroyl-L-aspartate (DDATH-PteAsp) was made by a modification of the procedure for the synthesis of DDATHF (1). (16)-Formyltetrahydrofolate (16-CHO-H₄PteGlu) was prepared from (16S)-3-formyltetrahydrofolate (3-CHO-H₄PteGlu) by first acidifying a solution to pH 1 for 20 min and then adjusting the pH to 8.0 for 30 min in the presence of 1% 2-mercaptoethanol. 10-Formyl-5,8-dideazafolic acid was a generous gift of Dr. Terence Jones (Argouron Pharmaceuticals, Inc.) and was used without further purification. The affinity column used to purify 5'-phosphoribosylglycinamid formyltransferase two absolute configurations of those naturally occurring reduced folates with a substituent at N-10 have the 6R-configuration, whereas those unsubstituted at N-10 have the 6S-configuration (3). The absolute configurations of DDATHF-A and B have recently been determined to be 6S and 6R, respectively, by unambiguous chemical synthesis from precursors of known stereochemistry (4). Thus, DDATHF-B and the naturally occurring tetrahydrofolate derivatives have the 6H on the same side of the ring.

1. The abbreviations used are: DDATHF, 5,10-dideaza-5,6,7,8-tetrahydrofolate; DDATH-PteAsp, 5,10-dideaza-5,6,7,8-tetrahydropteroyl-L-aspartate; 10-CHO-H₄PteGlu, 10-formyltetrahydrofolate; 5'-CHO-H₄PteGlu, 5-formyltetrahydrofolate.
2. The absolute configurations of those naturally occurring reduced folates with a substituent at N-10 have the 6R-configuration, whereas those unsubstituted at N-10 have the 6S-configuration (3). The absolute configurations of DDATHF-A and B have recently been determined to be 6S and 6R, respectively, by unambiguous chemical synthesis from precursors of defined stereochemistry (4). Thus, DDATHF-B and the naturally occurring tetrahydrofolate derivatives have the 6H on the same side of the ring.

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ase was made by linking 10-formyl-5,8-dideazafolic acid to cyanogen bromide-activated Sepharose using a two-carbon spacer as described by Young et al. (7). α,β-Glycinamide ribonucleotide was kindly provided by Dr. Homer Pearce (Eli Lilly & Co.) and was prepared by the method of Chettur and Benkovic (8).

**Cell Culture**—Mouse L1210 cells were continuously exposed to antiproliferative agents and protective agents for 72 h in RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum. The details of these procedures and the origins of the mouse L1210 leukemia and human lymphoblastic CEM cell lines have been previously described (2). Cell lines were found to be free of mycoplasma upon periodic assay.

**Folypolyglutamate Synthetase Assays**—Mouse liver folypolyglutamate synthetase was partially purified by (NH₄)₂SO₄ precipitation as previously described (9). These preparations had a specific activity of 0.8 IU/mg of protein. This enzyme had a specific activity of 1.2 nmol of folylpolyglutamate formed per h using 500 μM folic acid as substrate. We have previously found that these preparations were free of γ-carboxypeptidase activity and that the reaction catalyzed was linear with time and protein over the ranges used in this study. CEM cell folypolyglutamate synthetase was prepared by the same procedure. Reactions were incubated in 0.25 ml for 1 h at 37 °C in the presence of 1 mM [¹⁵N]glutamic acid, 5 mM ATP, 10 mM MgCl₂, and varying concentrations of folyl substrate in 200 mM Tris, pH 8.6, at 27 °C. Product was isolated and quantitated by the charcoal adsorption assay (10). Data were analyzed using the computer program of Cleland (11).

5′-Phosphoribosylglycinamide Formyltransferase—5′-Phosphoribosylglycinamide formyltransferase was purified from cultured L1210 cells by a modification of a procedure previously used for the purification of thymidylate synthase (EC 2.1.1.45) (12). Briefly, cytosolic protein was precipitated with 70% (NH₄)₂SO₄ and then dialyzed overnight. The resultant solution was applied to a 1 × 5-cm column of 10-formyl-5,8-dideazafolic acid-Sepharose. The column was washed extensively, and 5′-phosphoribosylglycinamide formyltransferase was eluted with (6R)-10-CHO-H₄PteGlu. Enzyme was passed through Sephadex G-50 equilibrated with 75 mM Hepes containing 20% (v/v) acetonitrile and then stored at −20°C. Denaturing polyacrylamide gel electrophoresis of pooled 5′-phosphoribosylglycinamide formyltransferase revealed a single band with a molecular mass of 110,000 daltons; a purity of >95% was estimated from these gels. When assayed at 25°C using 75 μM 10-CHO-5,8-dideaza-PteGlu and 200 μM α,β-glycinamide ribonucleotide as substrates, the enzyme had a specific activity of 0.8 IU/mg of protein.

In kinetic experiments with 5′-phosphoribosylglycinamide formyltransferase, reaction rates were measured at 25°C using the spectrophotometric assay that follows reaction at 298 nm using = ε₅₂₁ = 18,900 cm⁻¹ as described by Daubner and Benkovic (13). The buffer used in these experiments was 75 mM Hepes, pH 7.5, containing 20% glycerol and 45 mM 2-mercaptoethanol. The invariant substrate, aβ-glycinamide ribonucleotide as substrate, this enzyme had a specific activity of 0.8 IU/mg of protein.

**RESULTS**

**Separation of Diastereomers of DDATHF**—The 6R- and 6S-diastereomers of DDATHF were separated as the d-10-(+)-camphorsulfonic acid (CSA) salts as outlined in Scheme 1. When the N-2-acetyl diethyl esters of DDATHF (compound 1) were treated with 2 eq of CSA in refluxing absolute ethanol for 4 h and the mixture was allowed to cool, a crystalline mixture of salts separated which was composed of a 42:58 ratio of the CSA salts of the deacetylated diastereomers of the diethyl ester of DDATHF. After four fractional crystallizations of this material from absolute ethanol, one of the diastereomeric salts was obtained essentially pure (>97%) and was designated as CSA salt B, [α]₂⁰ = +20.02°. The CSA salt isolated by concentration of the mother liquors from the above initial crystallization proved to be enriched in the other diastereomeric salt, which was designated as CSA salt A (diastereomeric ratio A:B = 73:27). Repeated recrystallizations of this material from absolute ethanol at low temperatures (0–5°C) gave CSA salt A in >97% purity, [α]₂⁰ = +29.35°. The overall yield of CSA salt B was 15–20%, whereas CSA salt A was obtained in only 2–5%, based upon compound 1. Each of these diastereomeric salts was then saponified with 1.0 N NaOH in methanol. Diastereomer A of DDATHF, [α]₂⁰ = +31.09°, and diastereomer B of DDATHF, [α]₂⁰ = −21.06°, were obtained in quantitative yield.

Chiral column high performance liquid chromatography separated the diastereomers of DDATHF as the CSA salts of the diethyl esters, as the sodium salts, and as the free acids. When CSA salts A and B (see Scheme 1) were injected onto a 1.0 × 50-cm reverse-phase Cyclobond I column (which has β-cyclodextrin bound to the resin) and the column was eluted isocratically with 0.1% triethylamine/acetic acid, pH 7.0, containing 25% (v/v) acetonitrile at a flow rate of 1.5 ml/min, in a typical run, diastereomer A was retained for 37.1 min and diastereomer B for 39.5 min. The free acids of DDATHF-A and -B eluted at 21.3 and 24.8 min, respectively, in this system. This chromatographic system proved very useful for the analysis of stereochemical purity but was of limited value for the preparation of pure diastereomers due to the limited amount of material that could be applied to this column. The stereochirality of the material used in these experiments was >97% for both diastereomers by this criterion. Integration of the two peaks eluted after injection of unresolved DDATHF indicated that the molar ratio of diastereomers in the unresolved mixture was not detectably different from 50:50.

**Inhibition of Growth of Leukemia Cells by Diastereomers of DDATHF**—The growth inhibition caused by DDATHF (2) was reinvestigated using the resolved diastereomers. Both compounds were inhibitory to the proliferation of mouse L1210 leukemia cells (Fig. 1 and Table I). Hypoxanthine prevented the inhibition of growth of both diastereomers. Thymidine was without effect on the growth inhibition caused by either diastereomer in L1210 cells (Fig. 1 and Table I), as previously shown for the mixture of diastereomers (2).

The pattern of reversal of growth inhibition of diastereomers A and B by AICA in this cell line showed some differences. As shown by the data of Fig. 1A, AICA completely prevented the growth inhibitory effects of diastereomer A in L1210 cells up to concentrations as high as 10⁻⁴ M. AICA prevented the effects of concentrations of diastereomer B up to 10⁻² M; at higher concentrations, growth was progressively inhibited.
(6R)- and (6S)-DDATHF Inhibit Purine Synthesis

Inhibition of the growth of leukemia cells by the diastereomers of DDATHF and by a structurally related compound

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>n&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDATHF (mixture)</td>
<td>2.8 ± 1.0</td>
<td>14</td>
</tr>
<tr>
<td>DDATHF-A</td>
<td>2.9 ± 0.4</td>
<td>7</td>
</tr>
<tr>
<td>DDATHF-B</td>
<td>2.5 ± 0.9</td>
<td>6</td>
</tr>
<tr>
<td>DDATHF-A + dThd</td>
<td>3.0 ± 0.3</td>
<td>2</td>
</tr>
<tr>
<td>DDATHF-A + Hyp</td>
<td>&gt;10,000</td>
<td>3</td>
</tr>
<tr>
<td>DDATHF-A + AICA</td>
<td>&gt;10,000</td>
<td>4</td>
</tr>
<tr>
<td>DDATHF-B + dThd/Hyp</td>
<td>&gt;10,000</td>
<td>1</td>
</tr>
<tr>
<td>DDATHF-B + dThd</td>
<td>2.1 ± 0.7</td>
<td>2</td>
</tr>
<tr>
<td>DDATHF-B + Hyp</td>
<td>&gt;10,000</td>
<td>3</td>
</tr>
<tr>
<td>DDATHF-B + AICA</td>
<td>2,100 ± 3,000</td>
<td>3</td>
</tr>
<tr>
<td>DDATHF-PteAsp</td>
<td>87 ± 11</td>
<td>2</td>
</tr>
</tbody>
</table>

The concentrations of the protective agents were: dThd, 5.6 μM; Hyp, 100 μM; and AICA, 320 μM.

Values represent the number of determinations used to estimate the mean values.

Hyp, hypoxanthine.

Activity of the diastereomers of DDATHF for mouse liver folypolyglutamate synthetase

The kinetic constants were derived from weighted fit to a rectangular hyperbola using the program of Cleland (11). Substrate concentrations used were in the range of K<sub>m</sub> = 0.25–3, and there were about 10 points for each experiment.

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (μM)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate</td>
<td>49</td>
<td>140 ± 47</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>DDATHF (mixture)</td>
<td>5</td>
<td>7.8 ± 1.0</td>
<td>1.26 ± 0.07</td>
<td>30 ± 9.7</td>
</tr>
<tr>
<td>DDATHF-A</td>
<td>3</td>
<td>7.1 ± 1.0</td>
<td>1.16 ± 0.06</td>
<td>25.1 ± 2.3</td>
</tr>
<tr>
<td>DDATHF-B</td>
<td>3</td>
<td>9.3 ± 2.7</td>
<td>2.0 ± 0.13</td>
<td>28.7 ± 3.4</td>
</tr>
<tr>
<td>DDATHF-PteAsp</td>
<td>1</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values of K<sub>m</sub> were lower with human enzyme (1.0 and 1.7 μM, respectively, for diastereomers A and B), and substrate inhibition by diastereomer A was more pronounced (Fig. 2B, inset).<sup>3</sup>

This value was estimated using folypolyglutamate synthetase from CEM cells.

The level of substrate inhibition seen with unresolved DDATHF was intermediate between that seen with each diastereomer. Very similar results were obtained using folypolyglutamate synthetase partially purified from CEM cells (Fig. 2B). The only difference between the behavior of these compounds with mouse liver and CEM folypolyglutamate synthetases was quantitative: K<sub>m</sub> values were lower with human enzyme (1.0 and 1.7 μM, respectively, for diastereomers A and B), and substrate inhibition by diastereomer A was more pronounced (Fig. 2B, inset).<sup>3</sup>

The structurally related compound DDATH-PteAsp was not a substrate for folypolyglutamate synthetase at any concentration up to 500 μM (Table II) and was inhibitory to the growth of L1210 cells only at 30 times higher concentrations than those of DDATHF that were antiproliferative (Table I).

Folinic Acid Prevents Growth-inhibitory Effects of Individual Diastereomers of DDATHF—When leukemia cells were exposed to each of the individual diastereomers of DDATHF and to 5-CHO-H<sub>4</sub>PteGlu, the concentrations of DDATHF that caused inhibition were higher than those in the absence of 5-CHO-H<sub>4</sub>PteGlu (Fig. 3). Protection from the effects of DDATHF by 5-CHO-H<sub>4</sub>PteGlu was a linearly competitive phenomenon, i.e. for each 10-fold increment of 5-CHO-

<sup>3</sup>The level of substrate inhibition seen at higher concentrations of diastereomer B (Fig. 2) were attributable to the ~3% contamination with DDATHF-A.

Inhibited. Preformed purines (e.g. hypoxanthine) protected L1210 cells from high concentrations of diastereomer B in experiments in which AICA did not (Fig. 1B). This suggests that higher concentrations of diastereomer B were inhibitory to other steps in de novo purine synthesis, presumably 5'-phosphoribosyl-5-amino-4-imidazolecarboxamide formyltransferase (EC 2.1.2.3), as well as some other processes at very high concentrations.

Activity of DDATHF Derivatives as Substrates for Mammalian Folypolyglutamate Synthetase—Both diastereomers of DDATHF were found to be efficient substrates for folypolyglutamate synthetase of both mouse and human origin. As shown in Table II, the K<sub>m</sub> values found for diastereomers A and B were very similar for mouse liver folypolyglutamate synthetase and these values bracketed that found for the mixture of diastereomers. However, DDATHF-B had a higher V<sub>max</sub> value than diastereomer A in all three experiments with each enzyme. The first-order rate constant (V<sub>max</sub>/K<sub>m</sub> measured relative to folate in the same experiment) was not significantly different for the two diastereomers as a result of a balancing of small differences in K<sub>m</sub> and V<sub>max</sub>. The most distinct difference between the kinetics of folypolyglutamate synthetase with these two diastereomers was that diastereomer A partially inhibited the reaction at higher concentrations. This partial substrate inhibition was not observed with diastereomer B (Fig. 2A, inset).<sup>3</sup>
(6R)- and (6S)-DDATHF Inhibit Purine Synthesis

FIG. 2. Activity of diastereomers of DDATHF as substrates for human and mouse folylpolyglutamate synthetases. Partially purified folylpolyglutamate synthetase from mouse liver (A) or CCRF-CEM (B) cells was incubated with varying concentrations of each of the diastereomers of DDATHF, of the mixture of diastereomers (DDATHF), or of folate (PteGlu) for 1 h at 37 °C, and the product was isolated by charcoal adsorption. For details of the methods, see text. Each point represents the average enzyme velocity from two reaction mixtures.

H₄PteGlu added to the culture medium, a 10-fold higher concentration of DDATHF-A or -B was needed for growth inhibition. Reversal of DDATHF and of each of its diastereomers by 5-CHO-H₄PteGlu was equivalent in all respects studied (Fig. 3). However, the reversal of DDATHF by 5-CHO-H₄PteGlu was substantially more efficient than was the reversal of methotrexate by 5-CHO-H₄PteGlu, and the proportionality between concentration of drug that was inhibitory and concentration of 5-CHO-H₄PteGlu added to the culture medium was not observed with methotrexate under these conditions (Fig. 3). Thus, equimolar concentrations of 5-CHO-H₄PteGlu completely prevented the effects of either diastereomer of DDATHF, whereas at least a 400-fold molar excess of 5-CHO-H₄PteGlu was required to completely prevent the effects of 5 × 10⁻⁸ M methotrexate.

Inhibition of Mouse L1210 5'-Phosphoribosylglycinamide Formyltransferase by Diastereomers of DDATHF—The indirect evidence described above suggested that both diastereomers of DDATHF inhibited leukemic cell growth by interfering with a folate-dependent process early in de novo purine synthesis, presumably 5'-phosphoribosylglycinamide formyltransferase. This enzyme was purified from cultured mouse L1210 cells, and the interaction of the diastereomers of DDATHF with 5'-phosphoribosylglycinamide formyltransferase was studied directly. It was found that purification of this enzyme had to be complete to avoid proteolysis during incubation to fragments that retained catalytic activity but had altered kinetic properties, as has been previously noted by others (13, 14, 17). Enzyme used in our experiments retained a molecular mass of 110,000 daltons for at least 3 h of incubation at 25°C.

Each of the diastereomers of DDATHF was a moderately potent inhibitor of L1210 5'-phosphoribosylglycinamide formyltransferase (Fig. 4). Inhibition was competitive with the folate substrate used, 10-CHO-5,8-dideaza-PteGlu. Replots of the slopes of the lines from Fig. 4 against DDATHF concentration were linear for both diastereomers. Diastereomer A was a somewhat more potent inhibitor ($K_i = 0.029 ± 0.012 \mu M; n = 3$) than diastereomer B ($K_i = 0.10 ± 0.027 \mu M; n = 3$).

DISCUSSION

It is clear from our results that DDATHF is a mixture of two equipotent antifolates acting by very similar mechanisms. Both inhibit de novo purine synthesis. The data of Fig. 1 and
The specificity shown for DDATHF in Fig. 2. The difference in the phosphoribosylglycinamide formyltransferase (Fig. 2) is compatible with previous literature. Because of the proximity of the reactions catalyzed by a homogeneous preparation of 5'-phosphoribosylglycinamide formyltransferases by (6S)-10-CHO-H4PteGlu is in the absence of DDATHF (C) or in the presence of the indicated concentrations of diastereomers A (A) and B (B) of DDATHF.

Table I constitutes evidence that both diastereomers or their metabolites can interrupt the function of 5'-phosphoribosylglycinamide formyltransferase and, at high concentrations of DDATHF-B, 5'-phosphoribosyl-5-amino-4-imidazolecarboxamide formyltransferase in intact leukemic cells. These conclusions, which are based on cellular studies, are supported by the direct observation that both diastereomers of DDATHF are moderately potent inhibitors of pure L1210 5'-phosphoribosylglycinamide formyltransferase is remarkably minor.

Although we were initially surprised at the cytotoxic activity of both diastereomers of DDATHF, our results are compatible with previous literature. Because of the proximity of the center of asymmetry in DDATHF to the two positions (N-5 and N-10) of tetrahydrofolate involved in one-carbon transfer reactions, our expectation was that only one diastereomer of DDATHF would be active. However, the inhibition of mammalian (15) and bacterial (16) 5'-phosphoribosylglycinamide formyltransferases by (6S)-10-CHO-H3PteGlu is in accord with the concept that both diastereomers of antimeabolites of 10-CHO-H3PteGlu will bind to this enzyme. In addition, the low stereospecificity of mammalian folypolyglutamate synthetase for the naturally occurring diastereomer either of tetrahydrofolate (17, 18) or of 5'-CHO-H3PteGlu (19) would be in accord with the near complete lack of stereospecificity shown for DDATHF in Fig. 2. The difference in the potency of DDATHF-A and -B against 5'-phosphoribosylglycinamide formyltransferase is remarkably minor.

There are several indications that polyglutamate metabolites of the diastereomers of DDATHF will prove to be the active forms of these drugs. Thus, the low activity of DDATHF-PteAsp against leukemic cells in culture parallels its lack of activity as a substrate for folypolyglutamate synthetase. Although this fact seems to be an indication that polyglutamation plays a major role in the activity of this compound, we cannot rule out other possibilities, e.g. poor penetration of the cell membrane by DDATHF-PteAsp. Also, both diastereomers of DDATHF are inhibitory to intact cells at lower concentrations than would be expected from the strength of the interaction of this enzyme with the monoglutamate forms of these compounds. More recent results4 have indicated that the binding of polyglutamates of DDATHF-B to L1210 5'-phosphoribosylglycinamide formyltransferase is too tight to be quantitated by steady-state kinetics.

Thus, the cellular and biochemical data available on DDATHF make it clear that this compound represents a unique folate antimetabolite whose behavior furnishes novel insights into folate metabolism and chemotherapy.

Acknowledgments—We thank Drs. Homer Pearce and Terence Jones for their generous gifts of αβ-glycinamide ribonucleotide and 10-formyl-5,8-dideazafolic acid, respectively, and Dr. Stephen Benkovic for his helpful discussions. The technical expertise of Paul Colman, Valerie Evans, and Pamela Sartori made this work possible.

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